



## *Proteus vulgaris* and *Proteus mirabilis* Decrease *Candida albicans* Biofilm Formation by Suppressing Morphological Transition to Its Hyphal Form

Kyoung-Ho Lee, Su Jung Park, Sun Ju Choi, and Joo Young Park

Department of Microbiology, Yonsei University Wonju College of Medicine, Wonju, Korea.

**Purpose:** *Candida albicans* (*C. albicans*) and *Proteus* species are causative agents in a variety of opportunistic nosocomial infections, and their ability to form biofilms is known to be a virulence factor. In this study, the influence of co-cultivation with *Proteus vulgaris* (*P. vulgaris*) and *Proteus mirabilis* (*P. mirabilis*) on *C. albicans* biofilm formation and its underlying mechanisms were examined.

**Materials and Methods:** XTT reduction assays were adopted to measure biofilm formation, and viable colony counts were performed to quantify yeast growth. Real-time reverse transcriptase polymerase chain reaction was used to evaluate the expression of yeast-specific genes (*rhd1* and *rbe1*), filament formation inhibiting genes (*tup1* and *nrg1*), and hyphae-related genes (*als3*, *ece1*, *hwp1*, and *sap5*).

**Results:** *Candida* biofilm formation was markedly inhibited by treatment with either living or heat-killed *P. vulgaris* and *P. mirabilis*. *Proteus*-cultured supernatant also inhibited *Candida* biofilm formation. Likewise, treatment with live *P. vulgaris* or *P. mirabilis* or with *Proteus*-cultured supernatant decreased expression of hyphae-related *C. albicans* genes, while the expression of yeast-specific genes and the filament formation inhibiting genes of *C. albicans* were increased. Heat-killed *P. vulgaris* and *P. mirabilis* treatment, however, did not affect the expression of *C. albicans* morphology-related genes.

**Conclusion:** These results suggest that secretory products from *P. vulgaris* and *P. mirabilis* regulate the expression of genes related to morphologic changes in *C. albicans* such that transition from the yeast form to the hyphal form can be inhibited.

**Key Words:** *Candida albicans*, *Proteus vulgaris*, *Proteus mirabilis*, biofilm

### INTRODUCTION

Most microorganisms in natural ecosystems exist in the form of biofilms. The biofilms of microorganisms are formed over many stages, the first two of which are adherence to a biotic or abiotic surface and production of a structure to increase adherence. In the maturing biofilm, extracellular polymers are

formed by microorganisms in the structure and are known to protect microorganisms from changes in the surrounding environment, to participate in supplying nutrients and discharging metabolic waste, and to gather cells in closer proximity in order to facilitate cell-to-cell interactions. Biofilms can consist of multi-species, including coexisting bacteria and fungi.<sup>1-3</sup>

*Candida albicans* (*C. albicans*) is a resident species of healthy human mucous membranes that is also an opportunistic pathogen that induces superficial and systemic infection via the mucous epithelium when a patient suffers from severe disease or when the immune state is deficient. *C. albicans* has virulence factors that allow it to invade host tissue and to evade the host defense mechanism.<sup>4,5</sup> *C. albicans* grows in three different forms: budding yeast (or blastoconidia), pseudohyphae, and hyphae. The expression of *C. albicans* genes differ according to the *C. albicans* form. *Candida* hyphae are known to be essential to pathogenicity and disease dissemination,<sup>6-8</sup> and several

**Received:** July 11, 2017 **Revised:** August 21, 2017

**Accepted:** August 22, 2017

**Corresponding author:** Dr. Joo Young Park, Department of Microbiology, Yonsei University Wonju College of Medicine, 20 Ilsan-ro, Wonju 26426, Korea.  
Tel: 82-33-741-0322, Fax: 82-33-742-5034, E-mail: joopark@yonsei.ac.kr

•The authors have no financial conflicts of interest.

© Copyright: Yonsei University College of Medicine 2017

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

hyphae-specific genes are known, including *hwp1*, *als3*, *als8*, *ece1*, and *sap4-6*.<sup>9-11</sup> The yeast-specific genes include *rbe1*, *ywp1*, and *rhd1*, and filament formation inhibiting genes include *tup1* and *nrg1*.<sup>12-15</sup>

*Proteus vulgaris* (*P. vulgaris*) and *Proteus mirabilis* (*P. mirabilis*) exist in both human and animal small intestines and in the natural environment. *P. vulgaris* and *P. mirabilis* also can form biofilms on surfaces of various objects, including insertion apparatuses in humans. *Proteus* infections, especially urinary tract infections, are common in immunosuppressed patients.<sup>16-18</sup> In recent years, *C. albicans* has been highlighted as one of the most common etiologic agents of acquired hospital infections, and *Candida* biofilms play an important role in initiating infections. Biofilms that exist in the human body are comprised of hundreds of different bacterial species: fungi and bacteria can also co-exist in these biofilms.<sup>19</sup> Both the microorganism relationships with the host and the interactions between various microorganisms are important, the latter of which is not being actively researched.<sup>20,21</sup>

We previously reported that bacteria had a negative effect on the formation of *C. albicans* biofilms, and a more distinct decrease in *C. albicans* biofilm formation was shown when cultivated with *P. vulgaris*.<sup>22</sup> In the present study, the influence of co-culture of *C. albicans*, a chief agent of hospital-acquired infection, with *P. vulgaris* and *P. mirabilis* on the formation of *C. albicans* biofilm and its underlying mechanisms were examined.

## MATERIALS AND METHODS

### Organisms

Clinical isolates of *C. albicans* were obtained: one commensal strain was isolated from the blood of a patient, and *P. vulgaris* and *P. mirabilis* were isolated from the urine of another patient. The identity of each microorganism was confirmed with the commercially-available identification systems (BioMérieux, Marcy l'Etoile, France): API 32C for *C. albicans* and API 20E for *P. vulgaris* and *P. mirabilis*.

### Culture conditions and experimental conditions

Prior to each experiment, *C. albicans* isolates were cultured at 30°C for 48 hours on Sabouraud's dextrose agar (SDA, Difco™, Becton Dickinson, Spark, MD, USA), and one colony of yeast was inoculated into yeast nitrogen base (Difco™, Becton Dickinson) medium supplemented with 50 mM glucose. *P. vulgaris* and *P. mirabilis* were first subcultured at 37°C for 18 hours on tryptic soy agar. One colony each of *P. vulgaris* and *P. mirabilis* was then inoculated into tryptic soy broth (Difco™, Becton Dickinson) and incubated at 37°C for 18 hours. The experimental conditions were as follows: 1) the microorganism was cultured alone; 2) *C. albicans* was co-cultured with live *P. vulgaris* or *P. mirabilis*; 3) *C. albicans* was co-cultured with *P. vulgaris* or *P. mirabilis* killed at 100°C for 30 minutes; or 4) *C. al-*

*bicans* was treated with bacteria-cultured supernatants of *P. vulgaris* or *P. mirabilis* diluted four times, in which the bacteria were removed.

### XTT reduction assays

Biofilm formation was quantified using the method developed by Ramage, et al.<sup>23</sup> Biofilms were formed on commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Costar, Cambridge, MA, USA). Microorganisms were prepared for each condition and transferred to selected wells of a microtiter plate. The plate was incubated for 90 minutes at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cell suspensions were aspirated, and each well was washed twice with phosphate-buffered saline (PBS) to remove loose adherent cells. A volume of 200 µL of medium was added to each well, and the plate was then incubated for another 72 hours. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilm three times with PBS. A quantitative measure of biofilm formation was calculated using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay. A 200-µL aliquot of XTT (1 mg/mL, Sigma, St. Louis, MO, USA) and menadione (0.4 mM, Sigma) solution was added to each well containing the prewashed biofilm and the control well. The plates were then incubated in the dark for up to 3 hours at 37°C. A colorimetric change resulting from XTT reduction was measured using a microtiter plate reader (EMax, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

### *C. albicans* cell counts

After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilm three times with PBS. Then, 1 mL of PBS was transferred to each well, and biomass was meticulously scraped off. The resultant solution containing the detached biofilm cells was gently vortexed for 1 minute to disrupt the aggregates and inoculated on an SDA plate. The colony forming units (CFUs) of *C. albicans* were quantified after 48 hours of incubation at 30°C.

### Scanning electron microscopy

We developed biofilms from single species, in addition to *Candida* biofilms that were co-cultured with bacteria on polystyrene coverslips as described. The coverslips were washed twice with PBS and placed in PBS with a fixative of 2.5% glutaraldehyde (Sigma) for 20 hours. Next, they were washed for 5 minutes in PBS and then placed in 1% osmium tetroxide for 30 minutes. After a series of alcohol washes, a final drying step was performed using the critical point drying method. Biofilms were then mounted and gold coated. Samples were imaged with a scanning electron microscope (TM-1000, Hitachi, Tokyo, Japan) in a high-vacuum mode at 15 kV.

### Relative quantitation by real-time reverse transcriptase polymerase chain reaction

RNA was isolated from *C. albicans* cells using the MasterPure Yeast RNA Extraction kit (Epicentre Biotechnologies, Madison, WI, USA). RNA was treated with amplification grade DNase I (Epicentre Biotechnologies) and used for cDNA synthesis with random hexamer primer (Invitrogen Life Technologies, Carlsbad, CA, USA) using Superscript II reverse transcriptase reagents (Invitrogen Life Technologies). Each reaction contained 1 µg of total RNA, 1 µL of 50 µM hexamer, and 1 µL of 10 mM dNTP in a final volume of 10 µL. Reactions were incubated at 65°C for 5 minutes and cooled on ice. To each reaction tube, 10 µL of the following mixture was added: 4 µL of 5x First-Strand Buffer, 2 µL of 10 mM MgCl<sub>2</sub>, 2 µL of 0.1 M DTT, 1.4 µL of RNase inhibitor, and 1 µL of Superscript II. Reactions were incubated at 42°C for 50 minutes and then at 70°C for 15 minutes. Real-time polymerase chain reaction (PCR) contained 10 µL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), as well as forward and reverse primers (1 µL of each) (Table 1)<sup>22</sup> and sterile water, at a final volume of 20 µL. The PCR was run on MicroAmp® Optical 384-well reaction plates in an ABI 7900 Real-Time PCR system (Applied Biosystems). Real-time PCR reactions were performed at 95°C for 5 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Dissociation curves were analyzed for all reactions to verify single peaks/products. Expression levels were analyzed using ABI 7900 System SDS software (Applied Biosystems). Real-time PCR data were normalized with the geometric mean of two reference genes. The *ACT1* and *PMA1* genes were

used for this purpose.

### Statistical analysis

All experiments were performed in triplicate on three different occasions. All data are expressed as mean values with corresponding standard deviations (SDs). Student's t-tests and Mann-Whitney U-tests were used to compare the differences between *Candida* only and *Candida* co-cultured with *P. vulgaris* or *P. mirabilis*. All *p*-values <0.05 were considered statistically significant.

## RESULTS

### The effect of *P. vulgaris* or *P. mirabilis* on *C. albicans* biofilm formation

The biofilm value generated when each type of microorganism was incubated separately was 0.07±0.007 for *P. vulgaris*, 0.120±0.004 for *P. mirabilis*, and 1.403±0.103 for *C. albicans*. When culturing *C. albicans* and *P. vulgaris* or *P. mirabilis* together, biofilm formation was reduced by more than 80%, compared to the *C. albicans* culture alone. To assess the mechanism by which the bacteria impeded *Candida* biofilm formation, bacteria were initially treated for 30 minutes at 100°C to eliminate biological activity, followed by co-culture with *C. albicans*. Biofilm formation of *C. albicans* cultured together with heat-killed *P. vulgaris* or *P. mirabilis* elicited a 70% reduction, compared to the control group. These data indicate that dead bacteria interfere with the structural formation of *C. albicans* biofilms (Fig. 1A).

When *C. albicans* and *P. vulgaris* or *P. mirabilis* were co-cultured, biofilm formation of *C. albicans* was significantly reduced. In order to determine if this reduction was due to the effect of the bacteria or of secretory products when cultured, *P. vulgaris* and *P. mirabilis* were cultured for 72 hours, and remaining bacteria were removed by filtration. As a result of treatment with *P. vulgaris* and *P. mirabilis* culture supernatants, *C. albicans* biofilm formation was reduced by 60–70%, compared to *C. albicans* cultured alone (Fig. 1B). To determine whether this effect was due to a depletion of nutrients in the medium or to the secretory products of the bacteria, we diluted the cultured supernatants and tested the concentration effect on *C. albicans* biofilm formation. *C. albicans* biofilm formation decreased in proportion to the concentration of the bacterial-cultured supernatants (data not shown). To determine whether *P. vulgaris* and *P. mirabilis* inhibited the growth of *C. albicans*, *C. albicans* and *P. vulgaris* or *P. mirabilis* or bacterial-cultured supernatants were cultured together for 72 hours, and *C. albicans* CFUs were calculated. When *C. albicans* was cultured alone, the count was 2.85×10<sup>8</sup> CFU/mL; when cultured together with *P. vulgaris*, the count of *C. albicans* was reduced to 9×10<sup>6</sup> CFU/mL; and when *C. albicans* was cultured together with *P. mirabilis*, the count of *C. albicans* dropped to 2.4×10<sup>7</sup> CFU/mL (Fig.

**Table 1.** Primers Used for Real-Time RT-PCR

Gene	Primer	Product size
<i>sap5</i>	Forward 5'- CCAGCATCTTCCCGCACTT-3'	71 bp
	Reverse 5'-GCGTAAGAACCGTCACCATATTTAA-3'	
<i>als3</i>	Forward 5'-CAACTTGGGTTATTGAAACAAAAACA-3'	80 bp
	Reverse 5'-AGAAACAGAAACCCAAGAACAACCT-3'	
<i>ece1</i>	Forward 5'-CCAGAAATTTGTTGCTCGTGTG-3'	138 bp
	Reverse 5'-CAGGACGCATCAAAAACG-3'	
<i>hwp1</i>	Forward 5'- TCAGCCTGATGACAATCCTC-3'	105 bp
	Reverse 5'-GCTGGAGTTGTTGGCTTTTC-3'	
<i>rhd1</i>	Forward 5'-TTAGAGAAATGTGGCTGTGGTG-3'	155 bp
	Reverse 5'-TCACATAACCCTTTATCAGGCC-3'	
<i>rbe1</i>	Forward 5'-AGTAGTTCGGCCACTTCATATG-3'	175 bp
	Reverse 5'-GGAGTGCTCTCTTGACATTATG-3'	
<i>tup1</i>	Forward 5'-GCTTCAGGTAACCCATTGTTGAT-3'	88 bp
	Reverse 5'-CTTCGGTTCCTTTGAGTTTAGG-3'	
<i>nrg1</i>	Forward 5'-CACCTCACTTGCAACCCC-3'	198 bp
	Reverse 5'-GCCCTGGAGATGGTCTGA-3'	
<i>act1</i>	Forward 5'-ATGTGTAAGCCGGTTTTGCCG-3'	202 bp
	Reverse 5'-CCATATCGTCCCAGTTGGAAAC	
<i>pma1</i>	Forward 5'-ACTGCTGCCACAGTCAATGAAGC-3'	161 bp
	Reverse 5'-GCCATGTCACCACCACCGGA-3'	

RT-PCR, reverse transcriptase polymerase chain reaction.

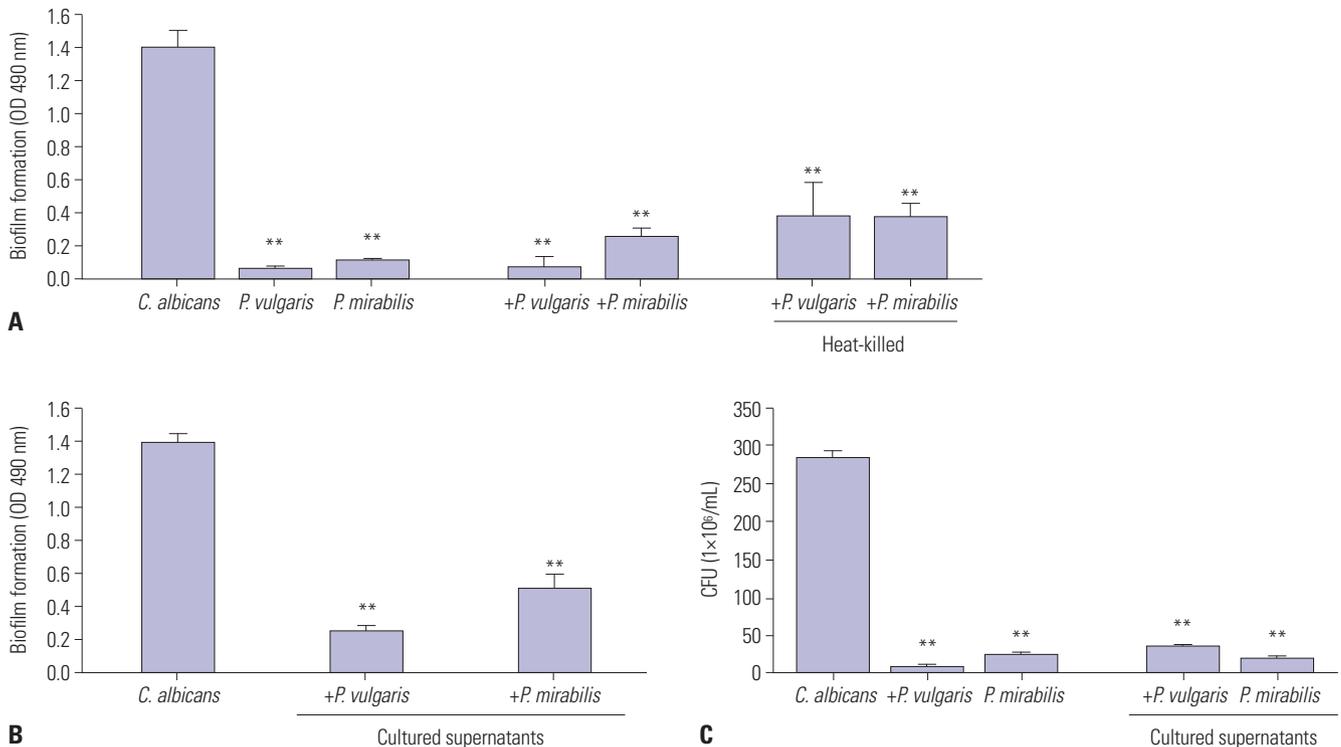
1C). It seems that *P. vulgaris* and *P. mirabilis* inhibit *C. albicans* biofilm formation and also interfere with its growth. As a result, we investigated the *C. albicans* CFUs after treating *C. albicans* with bacterial-cultured supernatants and culturing for 72 hours. In the case of culturing *C. albicans* alone, the count was  $2.85 \times 10^8$  CFU/mL. Meanwhile, the count from the treated culture supernatants of *P. vulgaris* was  $3.6 \times 10^7$  CFU/mL, and the count from the treated culture supernatants of *P. mirabilis* was reduced to  $2.0 \times 10^7$  CFU/mL (Fig. 1C).

**The effect of *P. vulgaris* or *P. mirabilis* on *C. albicans* morphology-related gene expression**

*C. albicans* morphology changes to the hyphae form from the yeast form as biofilm formation progresses, and the expression pattern of genes related to morphology at these stages was identified. Previous studies have shown that expression patterns of *Candida* species-related genes are significantly increased during hyphae-specific gene expression over the duration of biofilm formation. On the contrary, the expression of genes suppressing the formation of yeast-specific genes and filament are decreased.<sup>22</sup> These results suggest that *Candida* present in yeast form adhere to a surface to increase the expression of hyphae-related genes and promote the formation of biofilms by decreasing the expression of genes that inhibit the yeast form and filament formation. To clarify the effect of co-cultures on biofilm formation, we analyzed changes in *C. albi-*

*cans* gene expression levels in biofilms co-cultured with *P. vulgaris* or *P. mirabilis*. In contrast to *C. albicans* cultured alone, *als3* and *hwp1* showed a reduction in expression by 80% and *ece1* and *sap5* by 90% (Fig. 2A). When *C. albicans* was cultured together with *P. vulgaris* or *P. mirabilis*, the expression of *tup1* and *nrg1*, which are genes known to suppress filament formation, increased by about two-fold, compared to when *C. albicans* was cultured alone (Fig. 2B). Regarding the expression of yeast-specific *rhd1* and *rbe1*, the expression levels of *rhd1* increased by 2.5-fold and *rbe1* by about more than 3-fold (Fig. 2C).

When *C. albicans* was co-cultured with heat-killed *P. vulgaris* and *P. mirabilis*, biofilm formation was decreased. We confirmed that these results were due to changes in the expression of biofilm-related genes in *C. albicans*. First, *ece1*, *hwp1*, and *sap5* gene levels, which are associated with the formation of hyphae, did not increase. While *als3* level was slightly increased, the change was not significant (Fig. 3A). There were also no significant changes in *tup1* and *nrg1*, which are filament formation inhibiting genes (Fig. 3B). Yeast-specific genes also showed no difference in expression levels (Fig. 3C). The reduction in *C. albicans* biofilm formation in co-cultures with killed bacteria is considered to be caused not by changes in gene expression, but by the interference of killed bacteria acting as small particles that fit between *C. albicans* cells and, thus, suppress the structural formation of biofilm.

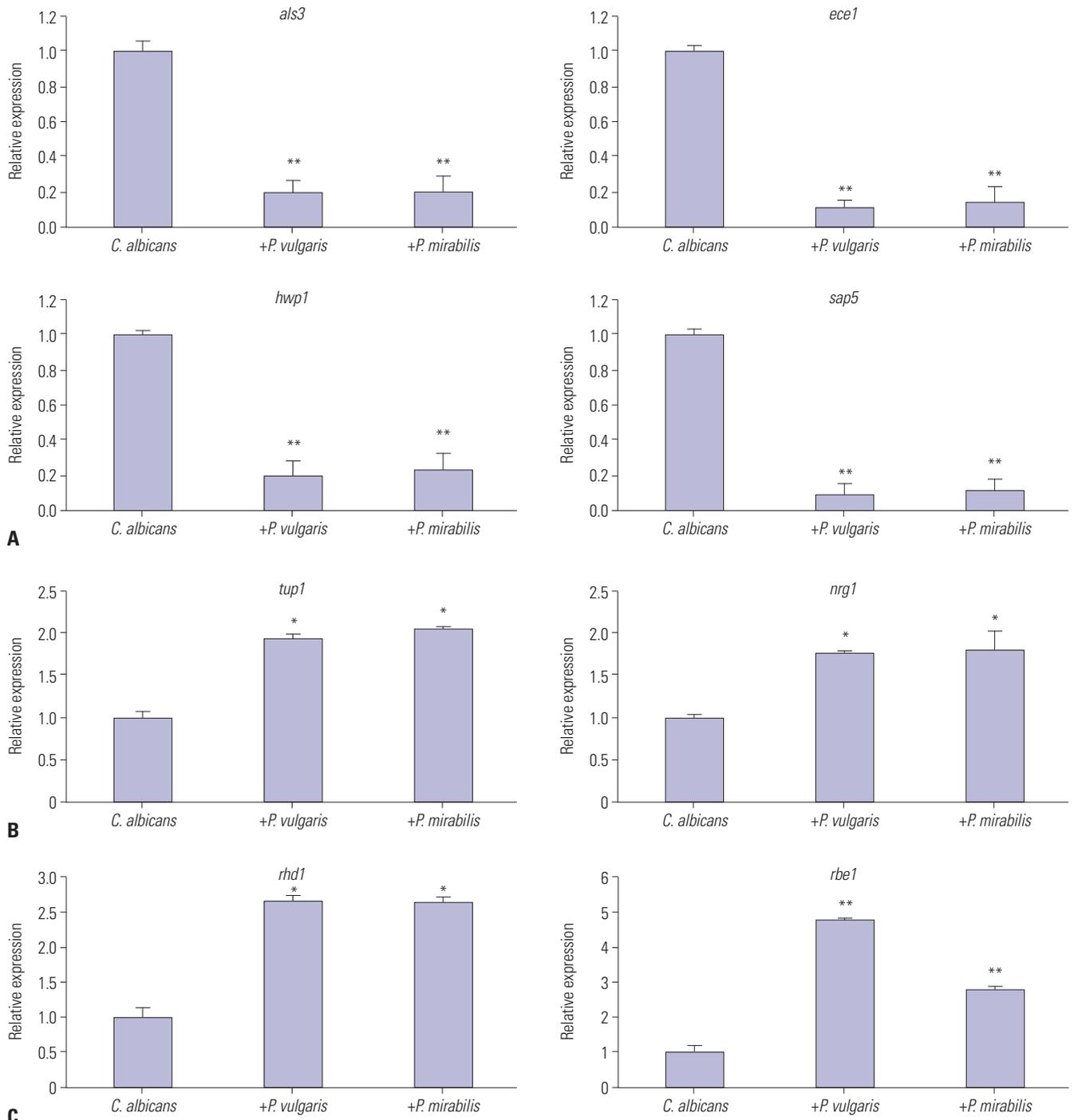


**Fig. 1.** The effect of *P. vulgaris* or *P. mirabilis* on *C. albicans* biofilm formation. (A) The co-culturing effect of live or heat-killed *P. vulgaris* and *P. mirabilis* on *C. albicans* biofilm formation. The effects of *P. vulgaris* and *P. mirabilis* cultured supernatant on biofilm formation (B) and growth (C) of *C. albicans*. The amount of biofilm formed was assessed with the XTT reduction assay. *C. albicans* cell counts were measured as colony forming units (CFUs) on a plate. The values of absorbance are the mean±SEM of three independent assays. \*\**p*<0.01 compared with biofilm formation of *C. albicans* only.

**The effect of cultured *P. vulgaris* or *P. mirabilis* supernatants on expression of *C. albicans* morphology-related genes**

As described above, treatment of *P. vulgaris* and *P. mirabilis* cultured supernatants inhibited *C. albicans* biofilm formation. We examined how this treatment affected the expression of

various genes involved in biofilm formation. The hyphae-specific genes *als3*, *ece1*, *hwp1*, and *sap5* all showed a significant reduction in expression in contrast to the cultured supernatants in which *C. albicans* was cultured alone (Fig. 4A). In contrast, the filament formation inhibiting genes *tup1* and *nrg1* slightly increased (Fig. 4B). The yeast-specific gene *rhd1* increased by



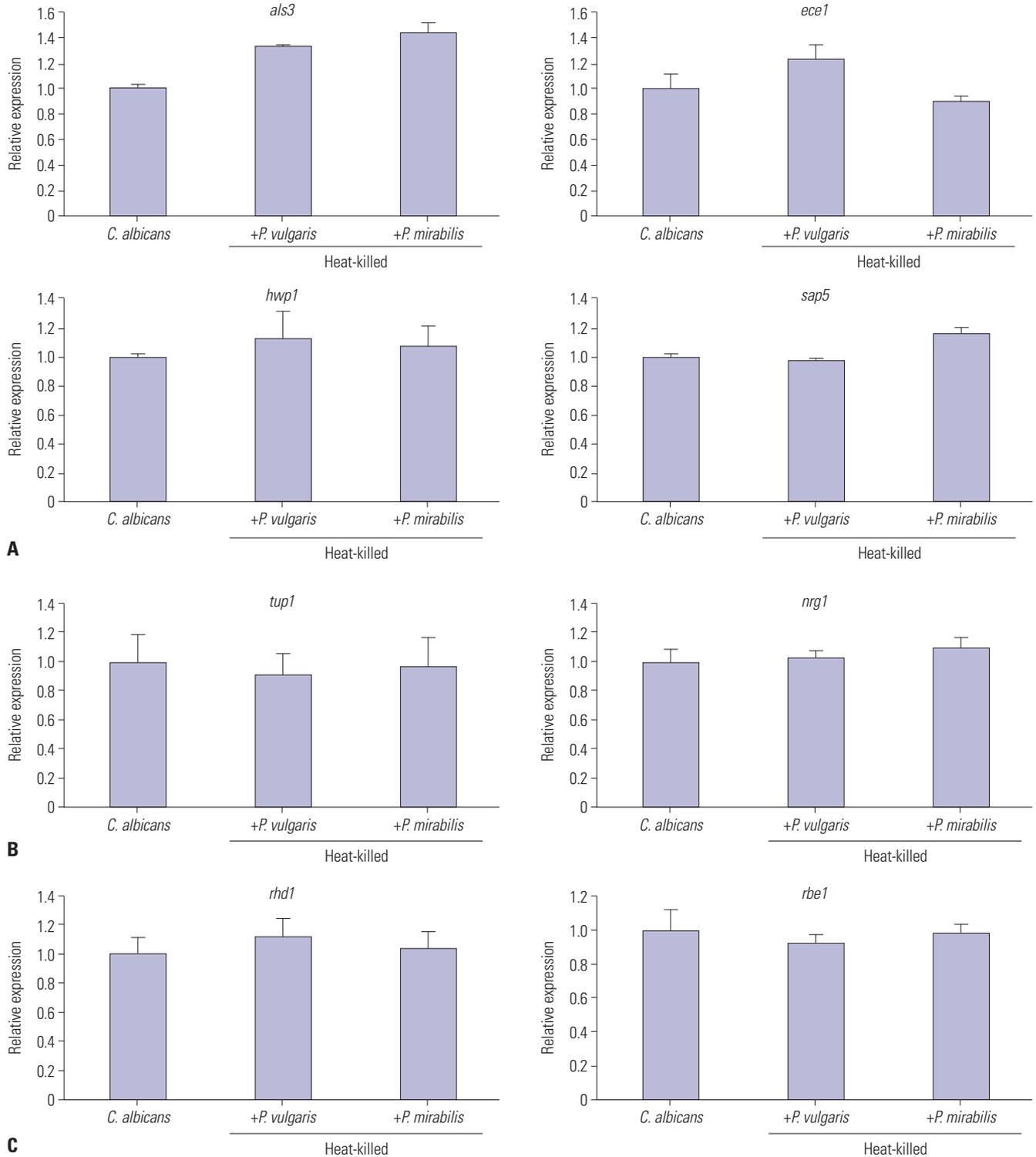
**Fig. 2.** The effects of live *P. vulgaris* or *P. mirabilis* cells on expression of *C. albicans* morphology-related genes. Relative quantitation of hyphae-specific (A), filament formation inhibiting (B), and yeast-specific (C) gene expression. Expression of genes was determined by quantitative real-time reverse transcriptase polymerase chain reaction. The data represent the average and standard deviation of three separate experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared with biofilm formation of *C. albicans* only.

2-fold, and *rbe1* slightly increased (Fig. 4C). This suggests that secretory products that are formed and released with growth of *P. vulgaris* and *P. mirabilis* inhibit the growth of *C. albicans* and regulate the expression of biofilm-related genes, thereby

inhibiting biofilm formation in *C. albicans*.

**Scanning electron microscopy of biofilms**

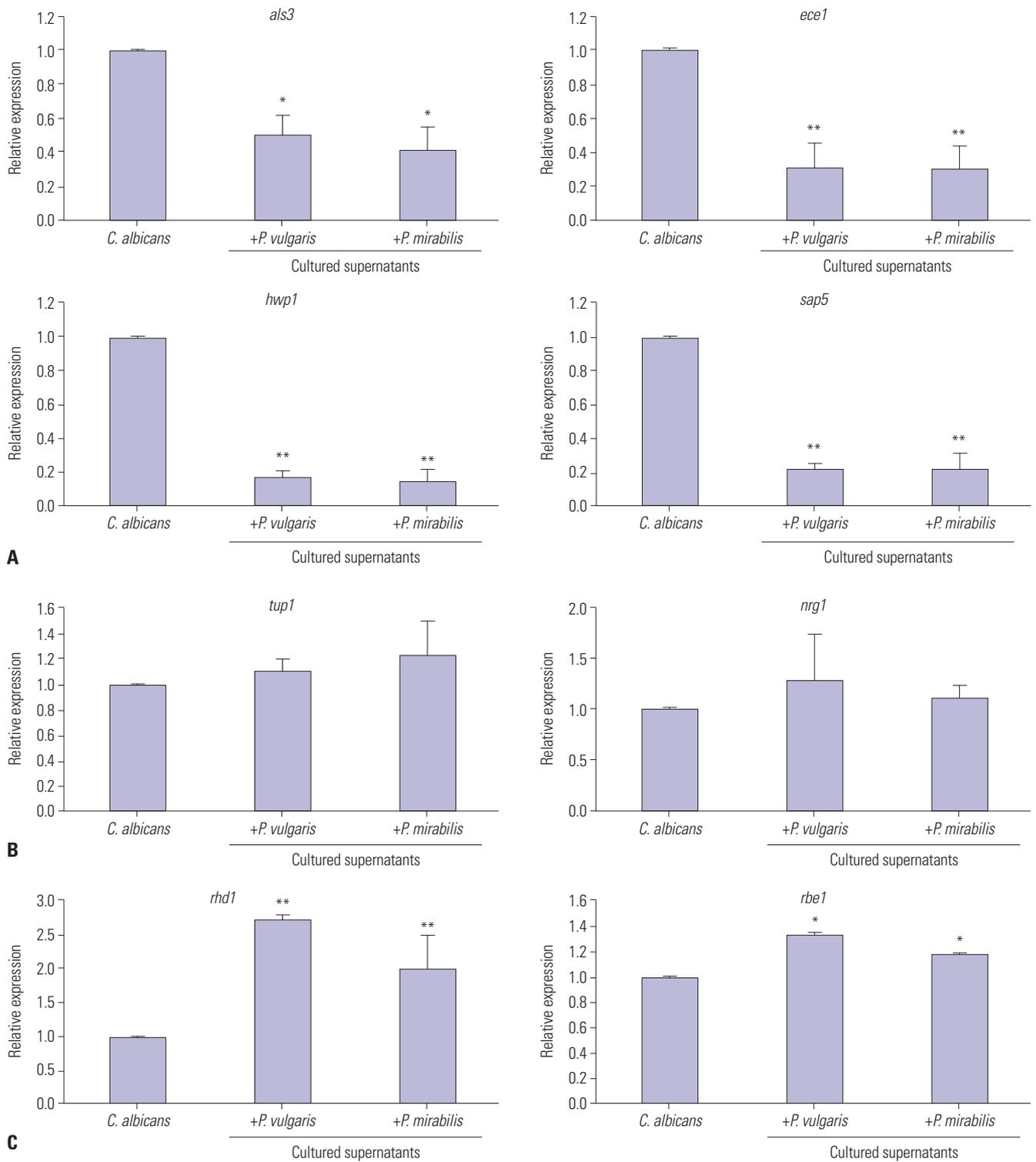
It is known that biofilms are not formed with a simple struc-



**Fig. 3.** The effects of killed *P. vulgaris* or *P. mirabilis* cells on expression of *C. albicans* morphology-related genes. Relative quantitation of expression of hyphae-specific (A), filament formation inhibiting (B), and yeast-specific (C) genes. Expression of genes was determined by quantitative real-time reverse transcriptase polymerase chain reaction. The data represent the average and standard deviation of three separate experiments.

ture and that *Candida* exist in the yeast form in the basal layer and in the hyphae and pseudohyphae form in the layer above. These layers form a network with a three-dimensional structure. The structural difference between the biofilm when *C.*

*albicans* is cultivated separately and when co-cultured with *P. vulgaris* or *P. mirabilis* was investigated via scanning electron microscopy (Fig. 5). The biofilm of *C. albicans* when cultured alone was high in density and was a multi-layer solid (Fig. 5A



**Fig. 4.** The effects of cultured *P. vulgaris* and *P. mirabilis* supernatants on expression of *C. albicans* morphology-related genes. Relative quantitation of hyphae-specific (A), filament formation inhibiting (B), and yeast-specific (C) gene expression. Expression of genes was determined by quantitative real-time reverse transcriptase polymerase chain reaction. The data represent the average and standard deviation of three separate experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared with the biofilm formation of *C. albicans* only.

and B). The biofilm formed when *C. albicans* and *P. vulgaris* or *P. mirabilis* were cultured together showed that the *P. vulgaris* or *P. mirabilis* were attached to the hyphae of *C. albicans* and inserted between the *C. albicans* cells. The thickness of the biofilm in these cases also appeared to be thin with a low density (Fig. 5C and E). The structure of biofilms formed after culturing heat-killed bacteria and *C. albicans* together was thinner and showed lower density than biofilms formed after separate culture of *C. albicans* (Fig. 5D and F). However, the heat-killed bacteria biofilm was thicker than the biofilm formed after *C. albicans* had been cultured together with live bacteria, and more of the *C. albicans* was in the yeast form. The above results agree with the outcome of XTT reduction assays, which quantitatively confirmed biofilm formation (Fig. 1A).

## DISCUSSION

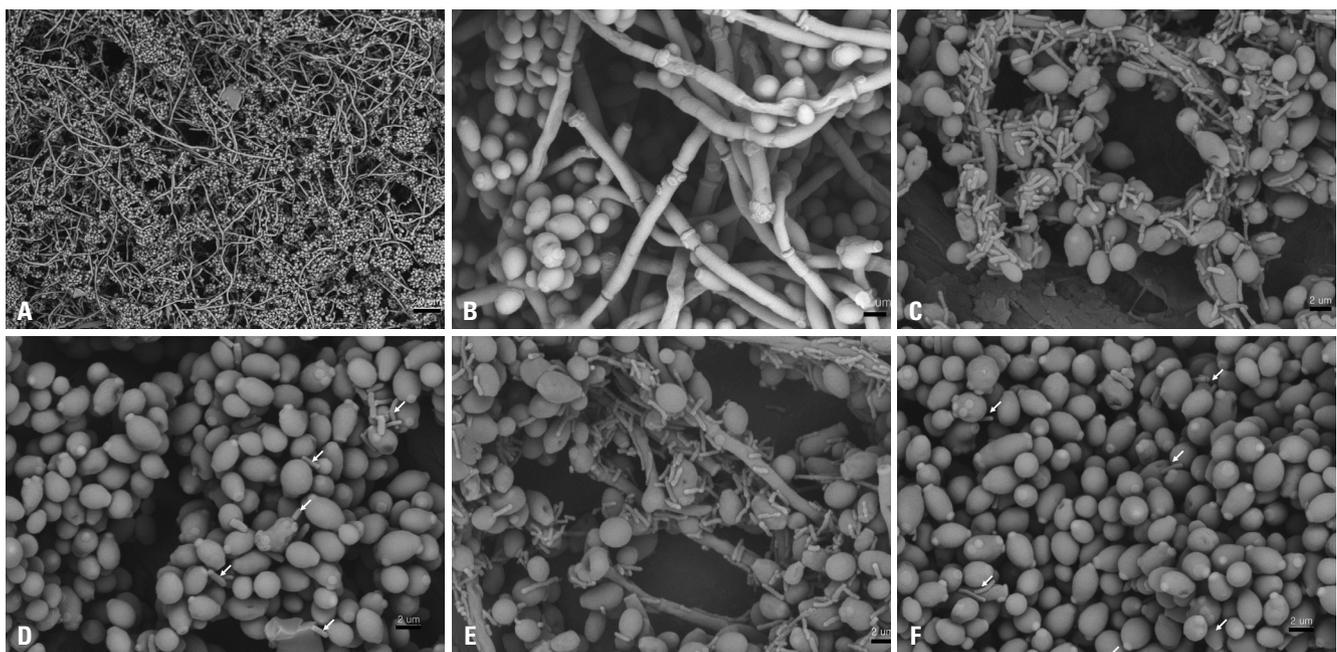
Microorganisms that are fixed inside a biofilm show resistance to the immune system and have a strong tolerance to antibiotics relative to planktonic microorganisms.<sup>24</sup> Many human infections are the result of microorganisms in biofilms. Most studies on biofilm formation and the interrelation between microorganisms in them have focused on bacteria.<sup>25</sup> However, biofilms affected by the relationship between bacteria and fungi are clinically crucial, because these biofilms increase the morbidity and mortality of infections.<sup>19,26</sup>

The research presented here verified the influence of the coexistence of *C. albicans* and *P. vulgaris* and *P. mirabilis* on *C. albicans* biofilm formation and whether the correlation be-

tween *C. albicans* and *P. vulgaris* and *P. mirabilis* was competitive or symbiotic. The architecture and functioning of complex biofilms are very intricate and were not clearly elucidated. In addition, the correlation between microorganisms inside the complex cultivated biofilm was not identified.

When *C. albicans* was cultivated with *P. vulgaris* and *P. mirabilis*, both biofilm formation and number of *C. albicans* cells decreased, compared to when *C. albicans* was cultured alone (Fig. 1). Even the diluted supernatants of *P. vulgaris* and *P. mirabilis* cultivation were confirmed to hinder biofilm formation, which implies that it was not the depletion of nutrients due to mixed culture of *P. vulgaris* and *P. mirabilis* plus *C. albicans* that decreased biofilm formation, but that the bacteria directly hindered formation due to secretory products (Fig. 1). The *Proteus*-specific products inhibiting the growth of *C. albicans* or biofilm formation are not yet clarified, and further research is needed.

Interestingly, even when *C. albicans* was cultivated with heat-treated *P. vulgaris* and *P. mirabilis*, biofilm formation decreased (Fig. 1A). It is considered that both the secretory products from *P. vulgaris* and *P. mirabilis* and the bacterial architecture itself induced structural changes and hindered the ability of *C. albicans* to form biofilms. Further, we verified biofilm structures of *C. albicans* alone or when cultivated with *P. vulgaris* and *P. mirabilis* via scanning electron microscopy. The biofilm formed normally only when *C. albicans* was cultured alone, showing high density and numerous layers (Fig. 5A). In contrast, biofilms that formed when *P. vulgaris* and *P. mirabilis* were cultured together showed low-density, thin biofilms with bacteria among the *C. albicans* cells, and a notice-



**Fig. 5.** Scanning electron microscopy of biofilms. *C. albicans* biofilm (A,  $\times 500$ ; B,  $\times 4000$ ). Biofilm of co-culture with *C. albicans* and live *P. vulgaris* (C) or heat-killed *P. vulgaris* (D); biofilm of co-culture with *C. albicans* and live *P. mirabilis* (E) or heat-killed *P. mirabilis* (F). Arrows point to examples of heat-killed bacteria. Magnifications are  $\times 500$  (scale bar; 20  $\mu\text{m}$ ) or  $\times 4000$  (scale bar; 2  $\mu\text{m}$ ).

ably decreased number of mycelia (Fig. 5). It is clear that both living and dead bacteria particles influenced structural maturation of the biofilm. Thus, *P. vulgaris* and *P. mirabilis* suppress the growth of *C. albicans* and can function as structural obstructive factors to the maturation of *Candida* biofilms.

The formation of hyphae is essential to *C. albicans* biofilm formation, so it is also important to understand the genetic basis of the morphological changes in *C. albicans*.<sup>10,11</sup> The mature biofilm enables the *Candida* yeast to fix the biofilm on to the extracellular surface, and the hyphae form a cross-sectional structure with structural frames.<sup>27</sup> Inhibition of the hyphae transgenes of *C. albicans* led to biofilm formation with the basal layer only, whereas inhibition of the yeast transgene of *C. albicans* led to biofilm formation with only the outer layer among the existing biofilm structures.<sup>11,28</sup> In this study, the expression of hyphae-related genes *C. albicans* was significantly inhibited in the presence of live *Proteus* or by *Proteus*-cultured supernatant. The expression of both yeast-related and filament formation inhibiting genes in *C. albicans* was up-regulated by treatment with live *Proteus* or *Proteus*-cultured supernatant (Figs. 2 and 4); however, the expression of morphology-related genes was not affected by heat-killed *P. vulgaris* and *P. mirabilis* (Fig. 3).

These results suggest that secretory products of *P. vulgaris* and *P. mirabilis* regulate the expression of genes that are related to morphologic changes, which could be the crucial factor in *C. albicans* biofilm formation, inhibiting hyphal transition from the yeast form to the hyphal form. Due to an increase in only the yeast form and the lack of hyphal form, the *C. albicans* biofilm would not form a solid 3D structure, but only a thick, basal-layered structure.

## REFERENCES

- Potera C. Forging a link between biofilms and disease. *Science* 1999;283:1837-9.
- Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002;15:167-93.
- Fuqua C, Winans SC, Greenberg EP. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* 1996;50:727-51.
- Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* 2000;13:122-43.
- Sugita T, Kurosaka S, Yajitate M, Sato H, Nishikawa A. Extracellular proteinase and phospholipase activity of three genotypic strains of a human pathogenic yeast, *Candida albicans*. *Microbiol Immunol* 2002;46:881-3.
- Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, et al. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* 2001;67:2982-92.
- Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol* 2003;11:30-6.
- Budtz-Jørgensen E, Mojon P, Rentsch A, Deslauriers N. Effects of an oral health program on the occurrence of oral candidosis in a long-term care facility. *Community Dent Oral Epidemiol* 2000;28:141-9.
- Staab JF, Bradway SD, Fidel PL, Sundstrom P. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* 1999;283:1535-8.
- Hoyer LL. The ALS gene family of *Candida albicans*. *Trends Microbiol* 2001;9:176-80.
- Liu H. Transcriptional control of dimorphism in *Candida albicans*. *Curr Opin Microbiol* 2001;4:728-35.
- Granger BL. Insight into the antiadhesive effect of yeast wall protein 1 of *Candida albicans*. *Eukaryot Cell* 2012;11:795-805.
- Sohn K, Urban C, Brunner H, Rupp S. EFG1 is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Mol Microbiol* 2003;47:89-102.
- Znaidi S, Nesseir A, Chauvel M, Rossignol T, d'Enfert C. A comprehensive functional portrait of two heat shock factor-type transcriptional regulators involved in *Candida albicans* morphogenesis and virulence. *PLoS Pathog* 2013;9:e1003519.
- Braun BR, Head WS, Wang MX, Johnson AD. Identification and characterization of TUP1-regulated genes in *Candida albicans*. *Genetics* 2000;156:31-44.
- Różalski A, Sidorczyk Z, Kotelko K. Potential virulence factors of *Proteus* bacilli. *Microbiol Mol Biol Rev* 1997;61:65-89.
- Jacobsen SM, Stickler DJ, Mobley HL, Shirtliff ME. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. *Clin Microbiol Rev* 2008;21:26-59.
- Jacobsen SM, Shirtliff ME. *Proteus mirabilis* biofilms and catheter-associated urinary tract infections. *Virulence* 2011;2:460-5.
- Thein ZM, Seneviratne CJ, Samaranyake YH, Samaranyake LP. Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* 2009;52:467-75.
- Adam B, Baillie GS, Douglas LJ. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J Med Microbiol* 2002;51:344-9.
- Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. *Sci Am* 1978;238:86-95.
- Park SJ, Han KH, Park JY, Choi SJ, Lee KH. Influence of bacterial presence on biofilm formation of *Candida albicans*. *Yonsei Med J* 2014;55:449-58.
- Ramage G, Vandewalle K, Wickes BL, López-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* 2001;18:163-70.
- Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol* 2005;13:34-40.
- Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2004;2:95-108.
- Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995;49:711-45.
- Hansen SK, Rainey PB, Haagenen JA, Molin S. Evolution of species interactions in a biofilm community. *Nature* 2007;445:533-6.
- Nett J, Andes D. *Candida albicans* biofilm development, modeling a host-pathogen interaction. *Curr Opin Microbiol* 2006;9:340-5.