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Induction of Mycelial Formation of *Candida albicans* by *Streptococcus salivarius* and Proline

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The aim of the present study was to clarify whether mycelial formation of *Candida albicans* was induced by bacterial cells of *Streptococcu salivarius* alone or by the joint effects of the bacterial cells and substances in the medium. Yeast cells of *C. albicans* were cultured in medium containing heat-killed *S. salivarius* bacteria or a heat extract of *S. salivarius* cells. The formation of mycelia of *C. albicans* was induced by medium containing intact *S. salivarius* cells or the bacterial extract in microwells. The mycelial formation of *C. albicans* needed both cell components of *S. salivarius* and proline in the medium. These findings indicate that the *S. salivarius* cells contain a substance that induces mycelial formation of *C. albicans* in a medium containing proline.

Key words : *C. albicans*, mycelia, *S. salivarius*, proline

Introduction

Candida albicans has been demonstrated to be a commensal microorganism in the oral cavity of healthy individuals, but its prevalence depends on age, habits and other factors. Regarding the morphotype of *C. albicans* detected in the healthy and diseased oral cavity, many yeast forms and many hyphal forms has been found[1], *C. albicans* is a dimorphic fungus that is classified based on the structural characteristics of the two forms (yeast and hyphae). The ability of *C. albicans* to undergo the yeast-to-hyphae transition is considered to enhance the pathogenicity by enhancing the invasion of *C. albicans* into human host tissues and its resistance against host defenses[2~5]. Though there have been various studies on factors that affect the induction of the hyphal formation of *C. albicans*[6~10], there have been few reports on the effects of bacterial species in the oral cavity on the hyphal formation. However, it has been reported that N-acetyl-D-gulucosamine, a component of the bacterial cell wall[9], and hemin, a component of the black-pigment of *Porphyromonas gingivalis*, affect the hyphal formation of *C. albicans*[11]. Accordingly, the aim of the present study was to examine whether the formation of candidal hyphae is affected by *S. salivarius*, a bacterial species. The rationale for this question is the fact that *C. albicans* is present on the dorsum of the tongues[1] and that *S. salivarius* is also predominantly present there in the oral cavity [12,13]. We studied whether candidal hyphal formation was induced by heat-killed *S. salivarius* bacterial cells or by a heat-extracted extract of the cells. The requirement for substances in the chemically defined medium used in this investigation was also examined.

Materials and Methods

1. Extract from bacterial cells and samples

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Cells of *S. salivarius* ATCC 7073 were aerobically propagated in tryptic soy broth medium (Difco Laboratories, Detroit, MI) at 37℃ for 72 h. The cells were separated from the medium by centrifugation and suspended in distilled water. Part of these intact bacterial cells was heat killed at 60℃ for 30 min and lyophilized. The remaining intact bacterial cells in distilled water were autoclaved at 120℃ for 90 min and the supernatant fluid was separated from the cells by centrifugation at 22200g for 10 min. The supernatant fluid was used as the extract from whole bacterial cells of *S. salivarius* ATCC 7073 and lyophilized, and the precipitate was used as the remaining fraction from the bacterial cells after extraction.

2. Culture in wells of microplates

Three samples : the unextracted whole bacterial cells, the extracted supernatant fluid and remaining bacterial cell fraction after extraction, were prepared at the desired dilution in distilled water, and 50 μl of each of these samples were added to the wells of 96-well/round bottom microplates. *C. albicans* NIH A207, which was examined for hyphal formation, was aerobically cultured on plates of Sabouraud medium for 18 h. The cultured yeast-form cells of *C. albicans* were suspended in distilled water (OD at 630 nm, 0.90) and diluted to a concentration of 9.5×10^6 cells per ml. Fifty microliters of the diluent was added to the wells of the microplate. Then, medium to be added to the wells was prepared by modification of a chemically defined medium described by Cho *et al*[6]. In this medium, $NaHCO_3$, CH_3COONa and Na_2HPO_4 salt solution concentrations were adjusted to 0.03 percent, and thioproline and proline were added to the solution at the same concentration. The final concentrations of these compounds in the medium in the wells was 0.01 percent, because the total amount of the medium in each well was 150 μl, including 50 μl of the chemically defined medium described above. The cultures were grown aerobically in a wet box containing the microplates at 37℃ for 4 days. In the study of the effects of components in the medium on hyphal formation of *C. albicans*, media supplemented with only the salt solution without either thioproline or proline, the salt solution including serial dilution of both thioproline and proline, and the salt solution containing either thioproline or proline, was used.

3. Examination by light microscopy

Mycelia were fixed by the addition of 50 μl of 40% formalin in medium in the 96 well microplate after culturing for 4 days. Morphological changes of *C. albicans* were observed and photographed under a phase contrast microscope at 10×magnification, and the thickness of the mycelia formed on and around the walls of the wells was measured using micrometer. Data about the thickness were expressed as the means of quadruplicate samples.

4. Statistics

Data were expressed as the mean±standard deviation. The results were evaluated by analysis of variance. Student's t-test was used for analysis of the difference between two groups.

Result

1. Mycelia formation of *C. albicans*

The effects of *S. salivarius* cells, proline and thioproline on mycelia formation of *C. albicans* were examined. *C. albicans* formed mycelia consisting of aggregates of hyphae. As shown in Fig. 1 A, two of the tested combinations promoted the formation of mycelia of *C. albicans*, namely, the combination of whole bacterial cells of *S. salivarius*, proline and thioproline, and the combination of the whole bacterial cells and proline. There was no significant difference between the effects of these two groups of factors on ability to form mycelia. The mycelia formed resembled

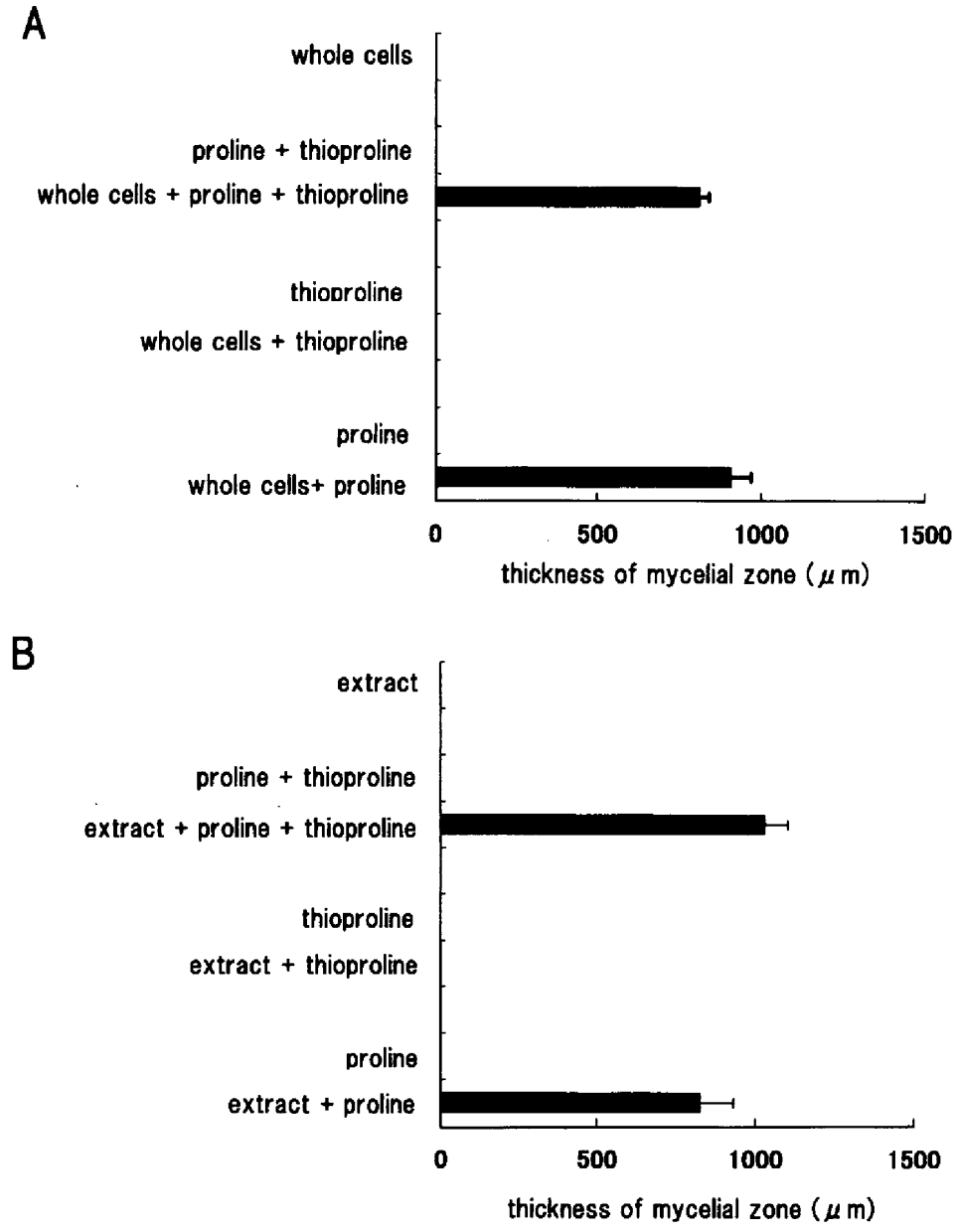

Fig. 1. Formation of mycelial zone of *C. albicans* around the inside walls of microwells. (A) The cultures of *C. albicans* were grown after addition of heat-killed bacterial whole cells (80 μg dry weight) of *S. salivarius* to chemically defined medium containing proline (120 μg) only, thioproline (120 μg) only, both these substances, or neither of these substances. (B) The cultures of *C. albicans* were grown after addition of extract (80 μg dry weight) from bacterial cells of *S. salivarius* to chemically defined medium containing proline (120 μg) only, thioproline (120 μg) only, both these substances, or neither of these substances.

hairy moss adhering to the wall of the wells, and the mycelia were thickly formed like a fence all around the wall of the well (These mycelia are referred to as the mycelial layer below). Next, in Fig 1 B, formation of the mycelial layer of *C. albicans* in the presence of the combinations of fac-

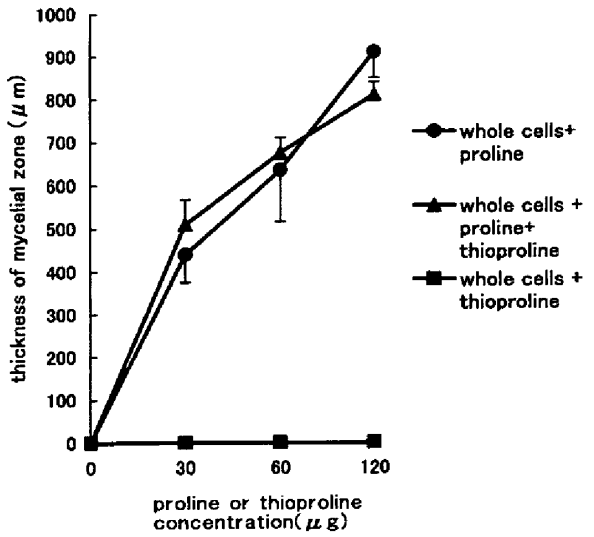

Fig. 2. The effect of variation of the proline or thioproline concentration on the formation of the mycelial zone of *C. albicans* around the inside walls of microwells. The cultures of *C. albicans* were grown after addition of heat-killed bacterial whole cells (80 μg dry weight) of *S. salivarius* to chemically defined medium containing only proline, only thioproline , both these substances, or neither of these substances.

tors including lyophilized samples of the extract from whole cells of *S. salivarius* is shown. In the case of the extract too, the formation of the mycelial layer of *C. albicans* was promoted by two combinations of factors : proline plus the lyophilized extract from the *S. salivarius* cells, and proline and thioproline plus the lyophilized extract. There was a significant difference between these two combinations of factors regarding the ability to promote the formation of the mycelial layer ($P<0.01$) .

2. Effects of concentration of proline and thioproline on formation of mycelial layer of *C. albicans*

Fig. 2 shows the formation of the mycelial layer of *C. albicans* in the wells when the concentrations of two components, proline and thioproline, described above was changed by serial two-fold dilution with an unchanged concentration (80 μg lyophilized weight) of whole cells of *S. salivarius*. The formation of the mycelial layer in the presence of the combination of whole cells of *S. salivarius* and proline or this combination plus thioproline was observed. In the latter treatment group, the concentration of thioproline had no effect on the formation of the mycelial layer, whereas the concentration of proline did affect it. The thickness of the mycelial layer of *C. albicans* formed in both treatment groups was the same. When proline was omitted from the medium containing *S. salivarius* whole cells and thioproline, formation of the mycelial layer was not observed at any concentration of thioproline. Fig. 3 shows the results of a study using an extract made from whole cells instead of whole cells of *S. salivarius*, as described above. Fig. 3A shows the formation of the mycelial layer of *C. albicans* when the concentrations of proline and thioproline were not changed as in the experiment shown in Fig. 2, except that *C. albicans* was treated with a constant amount of the extract (lyophilized weight 80 μg) of *S. salivarius*. *C. albicans* treated with either the combination of extract and proline or the combination of extract, proline and thioproline formed a mycelial layer in a manner dependent on the concentration of proline or of the mixture of proline plus thioproline. When using the extract of *S. salivarius*, there were a significant difference of the thickness of mycelial layer formed by *C. albicans* between the proline alone group and the proline plus thioproline group ($P<0.01$), although there was no such difference in the case of whole cells of *S. salivarius*, as described above. Namely, it was found that formation of the mycelial layer of *C. albicans* was enhanced by the addition of thioproline to proline plus the extract of *S. salivarius*. In contrast, when *C. albicans* was treated with the combination of the extract and thioproline without proline, a mycelial layer was not formed at any concentration of thioproline tested. These results show that

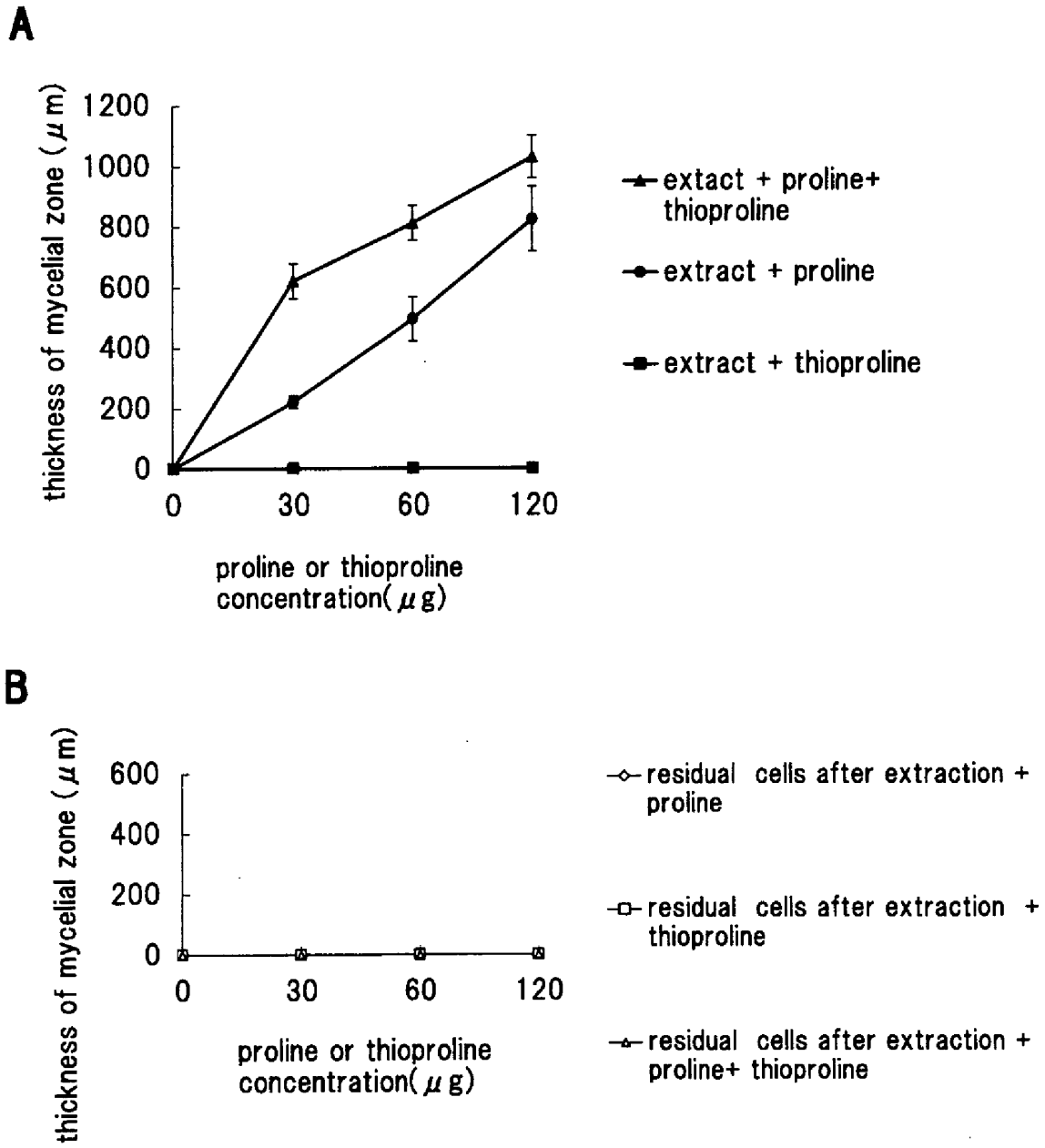

Fig. 3. The effect of variation of the proline or thioproline concentration on the formation of the mycelial zone of *C. albicans* around the inside walls of microwells containing bacterial extract from *S. salivarius*. (A) The cultures of *C. albicans* were grown after the addition of extract (80 μg dry weight) from bacterial cells of *S. salivarius* to chemically defined medium containing proline, thioproline, both these substances, or neither of these substances. (B) The cultures of *C. albicans* were grown after the addition of residual *S. salivarius* cells after extraction (80 μg dry weight) to chemically defined medium containing proline, thioproline, both these substances, or neither of these substances.

thioproline was less active than proline with respect to promoting mycelial layer formation. Fig. 3B shows the result of the test of the ability of the *S. salivarius* cells after extraction to promote mycelial formation. When the residual bacterial cells were used, no formation of a

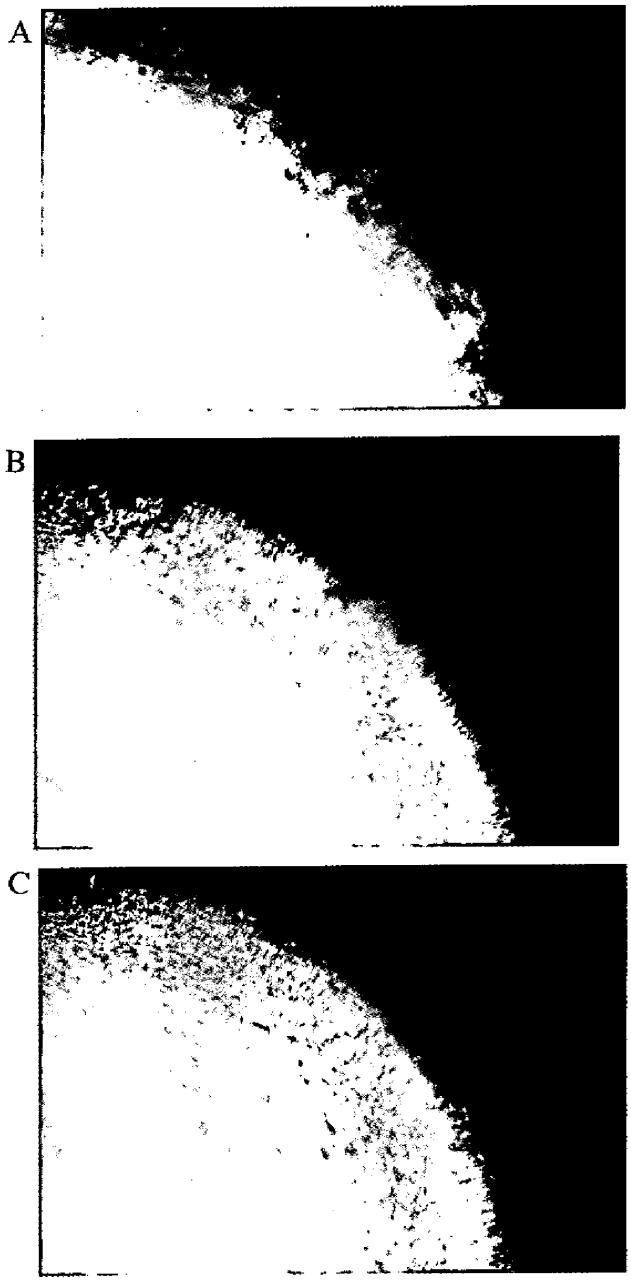

Fig. 4. Light micrographs of a part of the mycelial zone of *C. albicans* around the inside walls of microwells showing the effect of variation of the proline concentration on the formation of the mycelial zone of *C. albicans* in chemically defined medium containing extract from bacterial cells (80μg dry weight) of *S. salivarius*. Panel A 120 μg of proline ; Panel B 60 μg of proline ; Panel C 30 μg of proline. The original magnification of all micrographs was × 10.

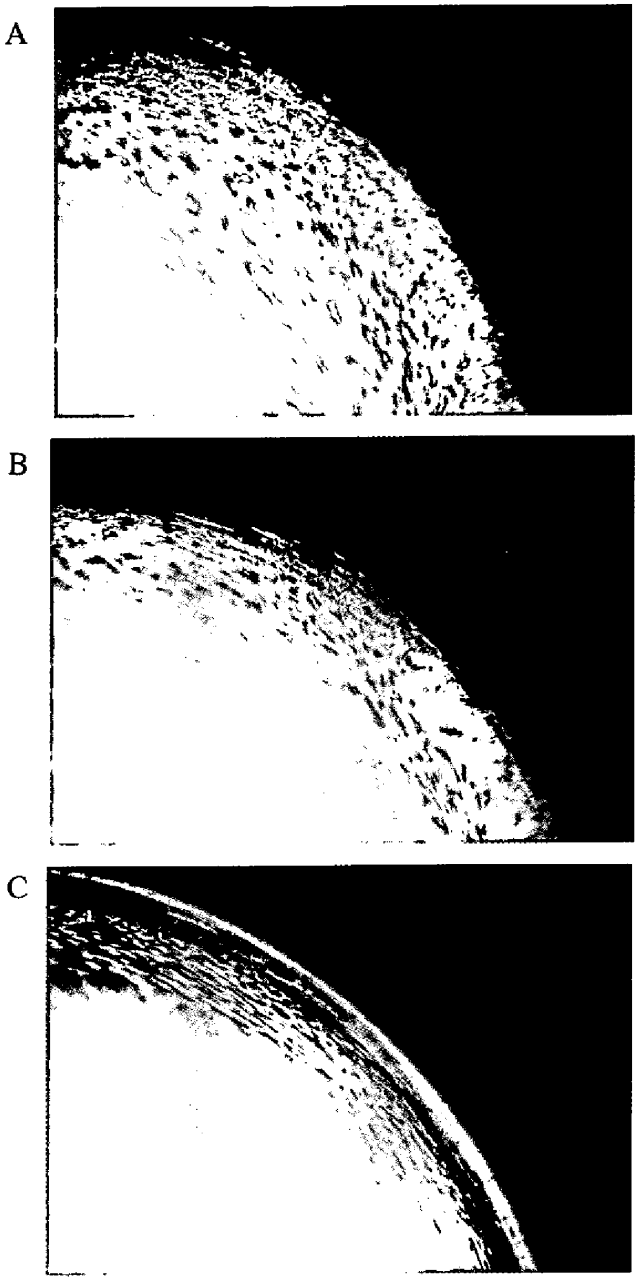

Fig. 5. Light micrographs showing lack of a mycelial zone of *C. albicans* around the inside walls of microwells. Panel A The inability of thioproline (120 μg) to promote the formation of a mycelial zone of *C. albicans* in chemically defined medium containing extract (80 μg dry weight) from bacterial cells. Panel B The mycelial zone of *C. albicans* was not formed in the chemically defined medium. The medium did not contain proline or thioproline but did contain extract (80 μg dry weight) from *S. salivarius* bacterial cells. Panel C The lack of formation of a mycelial zone of *C. albicans* in chemically defined medium that did not contain proline, thioproline or the extract from bacterial cells. The original magnification of all micrographs was × 10.

mycelial layer was observed with any of the combinations or concentrations of additions. This proved that the residual bacterial cells had no ability to promote the formation of the mycelial layer either alone or together with proline, because the substance in *S. salivarius* cells promoting the formation of the mycelial layer of *C. albicans* had been extracted into the extract of *S. salivarius*.

3. Light micrographs showing effects of the concentration of proline in the presence of the extract of *S. salivarius* cells

Fig. 4 shows light micrographs of the *C. albicans* mycelial formation in the presence of a constant amount of extract of *S. salivarius* (lyophylized weight 80 μg) supplemented with two-fold serial dilutions of proline. The micrographs show about one-fourth of each round well, and show the phase of the wall marginal zone in the wells. Panel A shows the thick mycelial layer formed in the presence of a high concentration of proline. This mycelial layer constructed a fence-like structure with long hypha elongating straight from the wall marginal zone of wells into the center of the well. The thickness of the layer reached over 800 μm. In panels B and C, the thickness of the mycelial layer decreased as the concentration of proline was reduced. This result shows that extract from *S. salivarius* cells promoted remarkable mycelial formation in cooperation with proline. In other experiments, under conditions that included proline, the extract from cells of *S. salivarius* and the unextracted whole cells similarly promoted the formation of the mycelial layer (not shown). The effect of proline is shown in panel A of Fig. 5. When proline was absent from the medium, formation of the mycelial layer of *C. albicans* was not observed even if the extract from cells of *S. salivarius* and thioproline were present. In the presence of the only the extract from cells of *S. salivarius* in salt solution (panel B) or only the salt solution (panel C), mycelial layer formation was not also observed. These findings show that proline was essential for formation of the mycelial layer.

4. Effect of the shape of the bottom of the wells on formation of mycelial layer of *C. albicans*

The mycelial layer that formed in the presence of the combination of the extract from cells of *S. salivarius* and proline was observed around the wall in the wells without well bottom. These findings were similar to the results obtained with the combination of whole cells of *S. salivarius* and proline. At 1 hour after the start of the culture, most of the whole cells of *S. salivarius* and the *C. albicans* yeast cells were precipitated on the round bottoms of the wells (not shown). Nevertheless, on the side of the wall of the round-bottom wells, a mycelial layer of *C. albicans* was formed after 4 days of culture. On the other hand, when flat-bottom wells were used, the mycelial layer was not formed on the side of the wall. Instead, the accumulation of long hyphae was observed on the flat bottom (not shown). These results suggest that the structural difference between the round bottom and the flat bottom of wells affected the formation of the mycelial layer due to a difference of the mode of convection of the medium in the wells.

Discussion

It was clarified in this study that the yeast-form of *C. albicans* gave rise to mycelia in a manner dependent on the presence of proline in the chemically defined medium used and on a bacterial components of *S. salivarius*. Both of these substances were essential for the formation of mycelia, because mycelial formation was not observed in the presence of proline only or the bacterial components of *S. salivarius* alone. Accordingly, I next discuss whether these two substances (proline and the bacterial compo-

nent) that participated in mycelial formation actually exist in the oral cavity. In a clinical report on the intra-oral cavity, hypha and mycelia were observed in stained preparations of scrapings from the lesions in chronic candidosis or in stained sections[14]. Penetration of hyphae was also observed in gingival tissue of HIV-infected patients[15,16]. As described above, firstly, there are reports on the formation of mycelia in or on oral mucosal tissue. Secondly, both *C. albicans*, a type of fungus, and *S. salivarius*, a type of bacterium, exist concurrently in microbial flora of the oral cavity. Therefore, it is suggested that these factors may contribute to the formation of hyphae or mycelia of *C. albicans* if proline, as the other essential factor, exists in the oral cavity, because *S. salivarius* is abundantly present on the mucosal membrane and tongue[17] and in saliva, and *C. albicans* also exists in those same areas[1,2]. Thirdly, regarding the possibility of a reciprocal action between proline and *S. salivarius*, both proline and proline-related substances have been shown to exist in the oral cavity. Proline-rich proteins (PRPs) contain large amounts of proline in saliva. Low molecular weight substances that include proline are the products of proteinase found in the saliva[18,19]. These substances may be converted to substance with even lower molecular weight by proteinase that *C. albicans* produces[20]. There is also proline in the collagen in periodontal tissue[21], and *C. albicans* can produce collagenase, which digests collagen[22]. Therefore, because there are proline sources in the oral cavity, *C. albicans* can probably use this proline. Accordingly, because bacterial cells of *S. salivarius* and proline, which were to promote formation of mycelia of *C. albicans* in the present study, have been confirmed to exist in the oral cavity in previous studies, future studies are needed to examine the participation of these facters in vivo. In addition, in the present study it was proved that the mycelial layer formation by *C. albicans* in the presence of proline and bacterial components of *S. salivarius* was affected by the shape of the well-bottom. When round-bottom wells were used, mycelial layers of *C. albicans* were formed around the side walls of the wells. However, when flat-bottom wells were used, deposits of long hyphae on the flat bottoms of wells were found, but mycelial layers were not formed around the side walls of the wells (not shown). From these findings, it was presumed that hyphal elongation starts from the walls, where the convection of the chemically defined medium favors the adherence of the yeast cells of *C. albicans* in the round bottom-wells. On the other hand, whole bacterial cells of *S. salivarius*, which were added as an inducer of the formation of the mycelial layer, were shown to be present in a precipitate in the round bottom wells. It is not clear how the mycelial layer formed at the wall in spite of the absence there of whole bacterial cells of *S. salivarius*, but these results suggest that soluble bacterial components extracted from whole bacterial cells of *S. salivarius* into the culture medium in round-bottom wells cause the *C. albicans* yeasts to adhere to the walls of the well. Regarding possible bacterial components, the strong formation of the mycelial layer of *C. albicans* was promoted either by a heat extract of *S. salivarius* or by whole bacterial cells of *S. salivarius*, in combination with proline (Fig. 1 A, B). On the other hand, the insoluble residual bacterial cells of *S. salivarius* after the active substances were extracted failed to promote mycelial formation of *C. albicans*. These residual bacterial cells were thought to consist of the cell wall, consisting of insoluble peptidoglycan as the basic structure. Peptidoglycan contains N-acetyl-D-glucosamine (GlcNAc) as its basic substances. It has been reported that formation of the germ tube of *C. albicans* was induced by GlcNAc[7]. Whether GlcNAc was released from peptidoglycan of *S.*

salivarius by treatment at high temperature or not in this study is unclear; however, the effect of GlcNAc on the formation of the mycelial layer of *C. albicans* was not thought to be major, because the combination of GlcNAc and proline did not promote the formation of the mycelial layer of *C. albicans* (data not shown). Accordingly, studies of other substances that may promote the formation of the mycelial layer of *C. albicans* are required.

In the present study, it was shown that *C. albicans* formed elongated hyphae from yeast type cells when incubated with proline and whole *S. salivarius* bacterial cells or extract from the whole *S. salivarius* cells, and then *C. albicans* formed a mycelial layer with a prominent aggregate on the walls of culture-plate wells.

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