Growth Inhibition of an Opportunistic Yeast Pathogen Trichosporon asahii by Staphylococcus epidermidis

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In the co-culture of Staphylococcus epidermidis and Trichosporon asahii, a fungal pathogen, it was observed that live S. epidermidis inhibited the growth of T. asahii. Soluble active anti-T. asahii substances were speculated to be produced by S. epidermidis in culture medium. Using 1H- and 13C-NMR spectra and electron ionization-high resolution mass spectrometry (HR-negative-FAB-MS), we separated the active molecule and identified it as lactic acid. Commercially available l-lactic acid and a-lactic acid inhibited the growth of T. asahii. These results show that metabolites from bacterial populations are involved in the interactions of pathogenic fungi. The use of antibacterial agents to treat primary diseases could lead to the disruption of normal microbial communities and could cause opportunistic infections such as trichosporonosis.

Key words Trichosporon asahii; Staphylococcus epidermidis; lactic acid; electron ionization-high-resolution mass spectrometry
of 100, 75, 50, 25, and 0% ethanol. The fraction, which was confirmed to inhibit the growth of \textit{T. asahii}, was concentrated and subjected to a reverse-phase cartridge column (Sep-Pack C18, Waters, U.S.A.) using a stepwise elution of 0, 25, 50, 75, and 100% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The active fraction in the pretreatment column was concentrated and further purified using a reverse-phase HPLC column (InertSustain, RP-C18, 4.6×250 mm) to identify the compound that inhibited the growth of \textit{T. asahii}. The peak associated with antifungal activity was collected, concentrated to dryness under a vacuum, and then subjected to 1H- and 13C-NMR spectral analyses and high resolution (HR)-negative-FAB-MS analysis to determine the structure of the antifungal component. 1H- and 13C-NMR spectra were recorded using a JEOL JMN-Lambda-500 spectrometer (1H: 500 MHz; 13C: 125 MHz). The chemical shifts are reported as $\delta$ values relative to the signal for the trimethylsilyl group in 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) at 0 ppm. HR-FAB-MS was performed with a JEOL JMS-700 double-focusing spectrometer.

Lactic Acid Determination \textit{S. epidermidis} was cultured in Sabouraud broth for 2d. The cell-free culture supernatant was isolated at 24 and 48 h by centrifugation (3500 rpm, 5 min) paired with filtration (0.45 $\mu$m). The supernatant was then used for determination of lactic acid. Lactic acid was measured using an HPLC method$^{[18]}$ with minor modifications. Briefly, the supernatant (5 $\mu$L) was injected onto an ODS-4 column (4.0×250 mm, GL Science, Japan), using 0.1% formic acid as the mobile phase at a flow rate of 0.8 mL/min. The eluent was monitored by UV-210 nm. l-Lactic acid was used as the standard.

RESULTS

Growth Inhibition of \textit{T. asahii} by \textit{S. epidermidis} The effects of \textit{S. epidermidis} on the growth of \textit{T. asahii} were examined using Sabouraud agar containing live cells of \textit{T. asahii}. The well was bored and 100 $\mu$L of \textit{S. epidermidis} culture was grown in nutrient broth at 37°C for 2d. As shown in Fig. 1A, an inhibitory zone of \textit{T. asahii} was observed near the well containing \textit{S. epidermidis}. When using Sabouraud agar containing phenol red, the inhibitory zone was determined to have an acidic pH (Fig. 1B). On the other hand, \textit{S. epidermidis} did not inhibit the growth of \textit{C. neoformans} B-3501 or \textit{C. albicans} under the same conditions (Figs. 1C, D).

Fractionation and Identification of the Anti-\textit{T. asahii} Substance We speculated that the unidentified substances inhibiting the growth of \textit{T. asahii} may be water-soluble molecules. Cell-free supernatant of \textit{S. epidermidis} culture was shaken with n-butanol, and the extract was concentrated to perform a growth inhibition test. As inhibition of \textit{T. asahii} growth was observed, the extract was applied to column chromatography. The fraction that was eluted by approximately 50% ethanol during silica gel column chromatography showed growth inhibition (Fig. 2A) because \textit{T. asahii} did not grow near the well containing this fraction. The fraction was separated using a reverse-phase cartridge column and the sample...
eluted with water exhibited inhibitory activity (Fig. 2B). This crude active fraction was further purified using a reverse-phase (C18) HPLC column with UV detection. As shown in Fig. 3A, many peaks were observed on the chromatogram; however, anti-\(T.\) asahii activity was only observed for a major hydrophilic peak (Peak 1) (Fig. 3B). Peak 1 was subjected to \(^1\)H- and \(^13\)C-NMR spectra and HR-negative-FAB-MS analysis, and lactic acid was identified as an antifungal component.

**Characterization Data** Isolated lactic acid: \(^1\)H-NMR (D\(_2\)O) \(\delta\): 1.36 (3H d, \(J=6.8\) Hz, \(-\text{CH}_3\)), 4.33 (1H, d, \(J=6.8\) Hz, \(-\text{CH(OH)}\)). \(^13\)C-NMR (D\(_2\)O) \(\delta\): 22.0 (q), 69.3 (d), 181.3 (s). HR-negative-FAB-MS (glycerol): \(m/z\) 89.0237 [M\(^{-}\)−H] (Calcd for C\(_3\)H\(_5\)O\(_3\): 89.0239). These data were identical to those of authentic L-lactic acid.

**Growth Inhibition Tests by Lactic Acid** Because lactic acid was identified as the anti-\(T.\) asahii active agent produced by \(S.\) epidermidis, growth inhibition tests were performed using commercially available L-lactic acid and D-lactic acid. As shown in Fig. 4, growth of \(T.\) asahii was inhibited by both undiluted (approximately 12 M) and 5-fold diluted lactic acid. Live \(S.\) epidermidis did not inhibit the growth of \(C.\) neofor-\(m\)\(a\)ns and \(C.\) albicans (Fig. 1), but lactic acid reagents did inhibit their growth, as shown in Figs. 5 and 6. However, the inhibitory zone was smaller in the case of \(C.\) neoformans and \(C.\) albicans.
**Determination of Lactic Acid in Culture Medium**

Lactic acid determination was performed using an HPLC-based method. The peak identified as lactic acid was not detected in Sabouraud broth before inoculation of *S. epidermidis*, but was detected in the culture supernatant after incubation. The amounts of lactic acid present after 24 and 48 h of incubation were calculated as 1.1 mg/mL (12.2 ms) and 2.4 mg/mL (26.6 ms), respectively.

**DISCUSSION**

Bacterial metabolites can be both useful and harmful to other living things. We purified and analyzed the molecule produced by *S. epidermidis* that inhibited opportunistic fungal pathogen, *T. asahii*, and identified it as lactic acid. We attempted quantitative analysis of lactic acid in the culture supernatant of *S. epidermidis* by HPLC. The contents in the medium were the same as in the inhibition assay, but without agar. In the medium prior to inoculation of *S. epidermidis*, lactic acid was not found, but it was determined and gradually increased in the medium after 24 h of incubation. Lactic acid diffused in agar, while *S. epidermidis* was not found, but it was determined and gradually increased in the medium. We attempted quantitative analysis of lactic acid in the culture supernatant, and identified it as lactic acid. Therefore, the anti-*T. asahii* activity of *S. epidermidis* is related to glycolysis and pyruvate metabolism. In *T. asahii*, a fungal pathogen, lactic acid might accumulate in addition to lactic acid. D-lactic acid did not have inhibitory activities against fungi, but lactic acid was identified in the active fraction prepared from the culture supernatant of *S. epidermidis*. It is possible that lactic acid is involved in the inhibition of *T. asahii* proliferation. In the human body, prevention of overgrowth of *T. asahii* by *S. epidermidis* on the skin may be possible. However, in the intestinal tract, lactic acid might be generated by lactic acid bacteria. Since lactic acid was identified as an inhibitor in this study, such bacteria would have the ability to control yeast pathogens.

The persistence of *S. epidermidis* as part of a normal bacterial microbiota would be helpful for defense against pathogens that cause opportunistic infection. Therefore, the use of antibacterial agents used in primary diseases could lead to the disruption of normal microbial communities.

**Conflict of Interest** The authors declare no conflict of interest.

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