Certain fungi, including those present in the domestic environment, cause various nasal, pulmonary, and skin diseases (Hay et al., 2016; Thompson and Patterson, 2012). In the past few years, fungal diseases reportedly have caused more than 1.6 million deaths annually, and 1 billion people are estimated to currently suffer from severe fungal diseases (Almeida et al., 2019; Brown et al., 2012). People with weakened immune systems or with serious illness need to take extra care to prevent fungal infections as they are at risk of worsening their conditions. Aspergillus and Cladosporium are among the most common indoor fungal species globally (Fukutomi and Taniguchi, 2015; Rosenbaum et al., 2010). Cladosporium is a strong risk factor for asthma that not only triggers but also aggravates asthma (Zureik et al., 2002). Sensitisation to Cladosporium has been found to be related to the aggravation of seasonal bronchial asthma (Pulimood et al., 2007). Aspergillus can cause various infectious and allergic diseases, depending on the host’s immune status or pulmonary structure (Kousha et al., 2011). In particular, A. fumigatus has a high capacity to colonise the bronchial tract of asthmatic patients, causing severe persistent asthma and declined lung function (Zureik et al., 2002). A. fumigatus can cause allergic bronchopulmonary mycosis, which can lead to bronchiectasis and pulmonary fibrosis if left untreated, as well as invasive pulmonary aspergillosis.

We previously identified a new Bacillus sporothermodurans strain, TM-I-3, which produces volatile compounds that show potent inhibitory activity against certain types of fungi, including Aspergillus fumigatus and Cladosporium cladosporioides. Non-contact antifungals derived from this bacterium may provide multidirectional inhibition and may be useful in disease prevention. This study is aimed at identifying the stage of fungal growth that is inhibited by TM-I-3 to elucidate the mechanism of its contact-independent antifungal activity. We evaluated mycelial growth and the gross fungal colony areas after 7 days in each experimental group varying the time in the proximity of TM-I-3 for 24 hours each. The fungal growth inhibition assay showed that TM-I-3 inhibited spore germination: the lag phase in the sigmoid growth curve. The present study demonstrated that TM-I-3 might be an effective fungistatic agent against pathogenic and allergenic fungi.

Key words: antifungal / Bacillus / fungistatic / volatile compounds.

Growth Inhibitory Mechanism of Contact-independent Antifungal TM-I-3 Bacillus sporothermodurans Strain against Aspergillus fumigatus and Cladosporium cladosporioides

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Note

We previously identified a new Bacillus sporothermodurans strain, TM-I-3, which produces volatile compounds that show potent inhibitory activity against certain types of fungi, including Aspergillus fumigatus and Cladosporium cladosporioides. Non-contact antifungals derived from this bacterium may provide multidirectional inhibition and may be useful in disease prevention. This study is aimed at identifying the stage of fungal growth that is inhibited by TM-I-3 to elucidate the mechanism of its contact-independent antifungal activity. We evaluated mycelial growth and the gross fungal colony areas after 7 days in each experimental group varying the time in the proximity of TM-I-3 for 24 hours each. The fungal growth inhibition assay showed that TM-I-3 inhibited spore germination: the lag phase in the sigmoid growth curve. The present study demonstrated that TM-I-3 might be an effective fungistatic agent against pathogenic and allergenic fungi.

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We previously reported the antifungal properties of a newly identified Bacillus strain, TM-I-3. Phylogenetic analysis revealed that this strain is closely related to Bacillus sporothermodurans (Osaki et al., 2019). We determined the properties and safety of this strain using genetic, morphological, physiological, and biochemical analyses. TM-I-3 was found to produce volatile compounds that are highly inhibitory to certain fungi, i.e., Aspergillus fumigatus, Cladosporium cladosporioides, and Penicillium expansum. These volatile compounds can be easily applied to fungal disease prevention in living spaces, for example by fixing a small well-ventilated container (including perlite inoculated with TM-I-3) to the wall of the bathroom or living room. Although bacterial strains with the same inhibitory characteristics have been reported previously, their mechanism of action has not been examined in detail and their safety for use has not been confirmed (Fiddaman and Rossall, 1993; Howell et al., 1988; Wright and Thompson, 1985). We expected our findings to lay a foundation for the application of TM-I-3 as a safe and effective antifungal agent for domestic use, and to provide further insight into the mechanisms underlying the interactions between Bacillus and fungi.

Thus, in this study, to elucidate the mechanisms of this contact-independent antifungal activity, we aimed at unravelling the stage of fungal growth inhibited by TM-I-3, using A. fumigatus and C. cladosporioides as representative pathogenic fungi.

B. sporothermodurans TM-I-3, isolated from soil in Nagasaki, Japan, was supplied by Mr. Ikari of T. M. Enterprise Co., Ltd. (Nagasaki, Japan). The bacteria were cultured in nutrient broth at 32 °C under shaking for 36 h. Perlite powder was then impregnated with the cultured bacterial strain and used for the inhibition assays. The fungal strains used in the experiments, A. fumigatus (NBRC 33022) and C. cladosporioides (NBRC 6348), were provided by the NITE Biological Resource Center (NBRC). Conidia of the two species were obtained as previously reported (Inouye, 2001). In brief, the strains were pre-cultured on potato dextrose agar (PDA) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 27 °C for 2 weeks. Fifteen mililitres of sterilised saline with 0.01% sodium dodecyl sulphate (Fujiﬁlm Wako Pure Chemical Corp., Osaka, Japan) was added to the plates for 3 min and then, conidia were scraped off using cell spreaders. The suspensions were ﬁltered several times through sterilised absorbent cotton to remove the mycelia. Conidia in the ﬁltrates were counted under a microscope, using a cell counter, and adjusted to 1.0 × 10^5–1.0 × 10^6 conidia/mL.

Prior to investigating the speciﬁc stage of fungal growth that TM-I-3 inhibits, we assessed the growth behaviour of A. fumigatus and C. cladosporioides. Potato dextrose broth (6.12 g potato dextrose powder [Becton, Dickinson and Company, Franklin Lakes, NJ, USA] in 300 mL of puriﬁed water) and conidial suspensions were mixed at a 1:1 ratio to yield a potato dextrose nutrient concentration of 0.01 g/mL. A modiﬁed version of Fujihiro’s method (Fujihiro, 2013) was used for continuous observation of changes in the conidia. In brief, 100 µL of PDA was spread on the bottom surface of a petri dish so as to prepare two squares (1 cm × 1 cm) of medium. One square of the medium of the same size was prepared with 200 µL of PDA for moisture retention. Ten microliters of the prepared potato dextrose broth were applied to each PDA square, and sterilized cover glasses (18 mm×18 mm, Matsunami Glass Ind., Ltd., Osaka, Japan) were placed on top. The plate was sealed with vinyl tape and incubated at 27 °C for a week. Fungal spore germination and mycelial growth were observed using a phase-contrast microscope (Olympus BX63; Olympus Corp., Tokyo, Japan), and digital images were acquired by the microscope’s digital camera (Olympus DP80; Olympus Corp.) every 24 h. Among the hundreds of images obtained, we selected those in which the individual hypha could be clearly distinguished. The mycelial length was measured from the digital images using cellSens Dimension ver. 1.16 and image editing software (Olympus Corp.). In a previous study, the parameter of the logistic sigmoid activation function was determined based on measurements, using a nonlinear least-square method (Ito et al., 2009). Using the parameter and the plotted

![FIG. 1. Structure of the experimental equipment using 5.6 L plastic boxes, used to identify the stage of fungal growth inhibited by TM-I-3.](image-url)
experimental data from this study, the fungal growth was modeled in Origin 2018b 64 Bit (LightStone, Tokyo, Japan).

To explore the fungal growth stage inhibited by TM-I-3, eight plastic containers (K-BOX; F-30, 5.6 L; 20.7 × 30.0 × 9.5 cm, ASVEL Co., Ltd., Nara, Japan) were prepared. A PDA plate containing 100 µL of conidial suspension of *A. fumigatus* or *C. cladosporioides* was fixed inside the lid of each container. For the inhibition assays, standard nutrient agar plates overlaid with 1 g of perlite in advance impregnated with TM-I-3, were incubated overnight at 32 °C. Following this incubation, plates with the bacterial suspension were placed at the bottom of each container (one plate per container) for 0, 24, 48, 72, 96, 120, 144 respectively (FIG. 1). Containers were incubated at 27 °C, and the control container had no agar plate. Gross fungal colony areas were measured using Foxit Reader®, 7 days after incubation.

FIG. 2 shows the time-course images of mycelial growth of *A. fumigatus* and *C. cladosporioides*. The images show that the hyphae started to extend from the spores of *A. fumigatus* and *C. cladosporioides* after 24 h and grew rapidly between 48 to 96 h of incubation. After 96 h, the hyphae produced new spores that were released. Arrow heads show the germinating spores, and hollow circles indicate the new spores.
growth of *A. fumigatus* and *C. cladosporioides*. Hyphae started to extend from the spores of *A. fumigatus* after 24 h of incubation and grew vigorously between 48 h and 96 h. After 96 h, new spores were produced and released. For *C. cladosporioides*, the hyphae extended from spores after 24 h of incubation and grew remarkably between 48 h and 96 h. After 96 h, the hyphae produced new spores at the tips, which were finally released.

In agreement with these observations, both the
species exhibited sigmoidal growth (FIG. 3). For A. fumigatus, the exponential phase started within 12 h after inoculation, and the stationary phase started at approximately 72 h (FIG. 3a). For C. cladosporioides, the exponential phase started at approximately 24 h after inoculation, and the stationary phase started at approximately 120 h (FIG. 3b).

The data from the fungal growth inhibition tests are shown in FIG. 4. Small A. fumigatus colonies could already be observed at 0 h (FIG. 4a); however, gross colony areas at 24 h and 48 h were two and three times greater, respectively. All three values (0, 24, and 48 h) were significantly different from the negative control, except those from 72 and 144 h (FIG. 4b).

For C. cladosporioides, none, or only a few colonies were observable at 0 h. Nevertheless, the gross colony areas at 24 h and 48 h were five and ten times greater than that at 0 h, respectively. These differences, however, were statistically significant only for 0 h, but not for 24 and 144 h (FIG. 4b).

The time-course of the fungal growth inhibition assay for A. fumigatus and C. cladosporioides revealed considerable (two to nine- and five to fifteen-fold) differences in the gross colony areas between 24 h of exposure to TM-I-3, and between 0 h of exposure to TM-I-3 and the negative control, respectively. This implies that TM-I-3 mostly affected the spores within 24 h after inoculation on PDA and thus effectively inhibited the growth of A. fumigatus and C. cladosporioides. In other words, TM-I-3 inhibited the lag phase within 0 to 24 h (sigmoid curves in FIG. 3a and 3b). Furthermore, FIG. 2 shows that some hyphae had germinated from the spores within 24 h after inoculation. Therefore, it is likely that TM-I-3 inhibits spore germination and exerts a contact-independent antifungal effect. On the contrary, it would be impossible for TM-I-3 to inhibit fungal growth after germination and extension or in the exponential growth phase. Thus, TM-I-3 might have fungistatic and preventive effects rather than microbicidal or sterilizing effects.

In conclusion, B. sporothermodurans strain TM-I-3 can inhibit the growth of human pathogens, including A. fumigatus and C. cladosporioides, without direct contact. TM-I-3 appears to exert fungistatic and preventive effects and might be applied to the indoor environment for the prevention of fungal infection. Given the potential value of this bacterial strain, its practical applications in living spaces are currently under investigation in our laboratory.

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