

Isolation and characterization of the polyhexamethylene biguanide hydrochloride-resistant fungus, *Purpureocillium lilacinum*

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Abstract

Polyhexamethylene biguanide hydrochloride (PHMB), an antimicrobial agent, has been widely used as a disinfectant in medical industries and public facilities. However, long-term use of any antimicrobial agent increases the risk of the microorganisms developing resistance. We aimed to examine the presence of microorganisms highly resistant to PHMB to address potential medical safety and public health concerns and devise strategies to prevent resistance development. We isolated and characterized a fungus from a 20% aqueous solution of PHMB and compared its microbiological characteristics and resistance profile with those of other major antimicrobial agents. Additionally, we sequenced the genome of the isolate to predict PHMB resistance-related genes. Based on the internal transcribed spacer (ITS) region of ribosomal DNA, the fungus was identified as *Purpureocillium lilacinum*. Although the *P. lilacinum* type and resistant strains showed similar morphology, the latter had extremely low PHMB susceptibility and was able to grow in the 20% aqueous solution of PHMB, which killed the type strain. Additionally, the minimum inhibitory concentration (MIC) of PHMB against the resistant strain was approximately four times higher than that against the type strain. In contrast, the MICs of four other antimicrobial agents and seven antifungal agents against the resistant strain were either less than or equal to those against the type strain. Furthermore, MICs of PHMB against four pathogenic filamentous fungi and two yeasts were significantly lower than those against the resistant strain. Despite its specific high resistance to PHMB, no gene homologous to fungal PHMB-resistant gene was found in the genome of the resistant strain. In summary, *P. lilacinum* was found to be significantly more resistant to PHMB than previously reported, suggesting an unidentified novel mechanism underlying drug resistance in the fungi.

1. Introduction

Antimicrobial agents are widely used to kill or suppress the growth of microorganisms. Polyhexamethylene biguanide hydrochloride (PHMB), a broad-spectrum antimicrobial and antiviral agent, is an effective disinfectant and biocide even when used at low concentrations. PHMB is active against both gram-positive (e.g., the minimal inhibitory concentrations (MICs) against *Bacillus subtilis* ATCC 6633 and methicillin-resistant *Staphylococcus aureus* NCTC 11940 are 5 and 2.5 µg/mL, respectively) and gram-negative bacteria (e.g., the MICs against *Escherichia coli* ATCC 9001 and *Pseudomonas aeruginosa* ATCC 25668 are 5 and 310 µg/mL, respectively) [1]. PHMB is also active against yeasts (e.g., the MIC₉₀ for *Candida albicans* isolates associated with infective keratitis is 1.56 µg/mL) [2], filamentous fungi (e.g., a 5 µg/mL PHMB treatment significantly inhibited arthroconidia germination and mycelial growth of *Geotrichum citri-aurantii*) [3], and viruses (e.g., PHMB was effective *in vitro* against herpes simplex virus type 1 at concentrations as low as 0.01%) [4]. Additionally, PHMB is water-soluble, odorless, colorless, thermostable, pH-stable, and presents low toxicity to mammals. Therefore, it is globally used in food, industrial, domestic, and institutional settings for hygienic purposes [5–7]. According to the manufacturer's recommendation, 20–200 and 200–400 µg/mL PHMB should be used for water treatment applications and the disinfection of pre-cleaned solid surfaces, respectively (Arch Chemicals, Inc., Norwalk, CT, USA, 2005) [8].

It is important to select an antimicrobial agent based on its antimicrobial spectrum and consider concentration, pH, contact time, and temperature suitable for use. Inappropriate and excessive use of antimicrobial agents has led to concerns regarding microbial contamination in various industries and the emergence of drug-resistant, clinically relevant pathogens. Makela *et al.* reported that the lowest concentrations of commercial germicides recommended by manufacturers are not sufficient to disinfect the slime-producing lactic acid bacteria in the rooms and on the equipment of meat-processing plants [9]. Henly *et al.* demonstrated that long-term exposure of uropathogenic *E. coli* to commonly used biocides lowers its susceptibility to these agents, which may be explained by phenotypic alterations impacting biofilm formation, antibiotic susceptibility, and relative pathogenicity [10]. Thus, the emergence of antimicrobial resistance and contamination by resistant microorganisms is a critical issue.

It has been proposed that PHMB adheres to the cell membrane and exerts its antimicrobial activity by disrupting membrane structure, mainly in prokaryotic cells. Recently, Michael *et al.* demonstrated that a direct and strong cooperative binding between PHMB and genomic DNA leads to bacterial cell death, presenting a new theory explaining the mechanism of action of PHMB [11]. However, this theory does not apply to eukaryotic cells that have more extensive compartmentation of genomic DNA, in which internal structures act as barriers blocking the direct interaction between PHMB and DNA. Elsztein *et al.* examined the cellular mechanisms responsible for low susceptibility in the PHMB resistant *Saccharomyces cerevisiae* strain JP-1 [12], and provided biological evidence supporting the participation of the NCW2 gene encoding novel cell wall protein 2 in the mechanism responsible for cell tolerance to PHMB [13]. The NCW2 gene was found to be among the cell wall integrity genes of *S. cerevisiae*, and its protein had an auxiliary function in the maintenance of the glucan/chitin balance and ensuring the correct structure of the yeast cell wall [14].

By serendipity, we noted white floating masses in a 20% aqueous stock solution of PHMB. The mass showed mycelial morphology under a microscope, and we isolated a fungus from the contaminated PHMB solution. This study aimed to investigate the microbiological characteristics of the isolated high PHMB-resistant fungus and its resistance profile against major antimicrobial agents, to understand the clinical implications and public health concerns associated with such resistant fungi. Furthermore, we sequenced the genome of the isolate to capture its genome features and predict PHMB resistance-related genes, referring to the previously reported NCW2 gene sequence. The findings will also be applied in future to prevent the emergence of PHMB-resistant fungi.

2. Materials And Methods

2.1. PHMB aqueous solution

The PHMB aqueous solution used for fungal detection was obtained by repackaging a sample from Vantocil™ IB (Lonza, Basel, Switzerland), a 20% aqueous solution of PHMB, aliquoted into 20-L polyethylene containers, and was stored statically at 20–25 °C for more than 1 month.

Each concentration of PHMB aqueous solution for susceptibility testing was prepared by diluting Vantocil™ IB with sterile ion-exchanged water.

2.2. *P. lilacinum* strains and culture conditions

P. lilacinum IFM 47467^T (NBRC 5350^T), NBRC 5752, NBRC 31847, NBRC 31914, NBRC 31970, NBRC 32861, and NBRC 103232 were obtained from NITE Biological Resource Center (Tokyo, Japan), and *Aspergillus fumigatus* IFM 54229 (= Af293), *Aspergillus niger* IFM 62238, *Fusarium solani* IFM 62065, *Penicillium citrinum* IFM 62178, *Candida albicans* IFM 40009, and *Cryptococcus neoformans* IFM 46660 were obtained from the Medical Mycology Research Center (Chiba University, Chiba, Japan) through the National Bio-Resource Project of the MEXT, Japan (Table 1). The stock cultures were revived under the conditions recommended by the respective culture collections. The working cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. The strains were cultivated on PDA plates at 22.5–25 °C until sporulating colonies of appropriate size were observed.

Table 1
List of culture strains used in the present study

Scientific name	Culture No.	Source
<i>Purpureocillium lilacinum</i>	IFM 47467 ^T (NBRC 5350 ^T)	Soil
	NBRC 5752	Unknown
	NBRC 31847	Paddy field soil
	NBRC 31914	Silk-worm food
	NBRC 31970	Soil
	NBRC 32861	Living leaf, <i>Phragmites karka</i>
	NBRC 103232	Collembolan, Arthropleona, Collembola
<i>Aspergillus fumigatus</i>	IFM 54229	Clinical specimen
<i>Aspergillus niger</i>	IFM 62238	Otorrhea
<i>Fusarium solani</i>	IFM 62065	Clinical specimen
<i>Penicillium citrinum</i>	IFM 62178	Scalp
<i>Candida albicans</i>	IFM 40009	Patient with pulmonary candidiasis
<i>Cryptococcus neoformans</i>	IFM 46660	Pulmonary tissue

2.3. Fungal isolation

White floating masses were collected from a 20% aqueous solution of PHMB with a micropipette and were morphologically examined under a light microscope (ECLIPSE Ni-U; Nikon Co., Tokyo, Japan) equipped with a digital camera (DS-Fi2; Nikon Co., Tokyo, Japan). Mycelia were inoculated on PDA plates

and incubated at 22.5 °C for 6 days. Single colonies were re-inoculated three times on new PDA plates to obtain pure isolates.

2.4. Identification of fungal isolates

The genomic DNA of the isolates was extracted by physical disruption and using Marmur's method with some modifications [15]. The complete nuclear ITS region, including ITS1, 5.8S, and ITS2, was PCR-amplified using specific primers ITS5 [16] and NL4 [17] and PrimeSTAR HS DNA Polymerase (Takara Bio Inc., Shiga, Japan). The PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The complete nuclear ribosomal ITS regions were sequenced using ITS5 and ITS4 primers [16]. The sequences were assembled using ChromasPro 1.7 (Technelysium Pty, Ltd., Tewantin, QLD, Australia). Multiple sequence alignments were performed using CLUSTAL W [18], and the final sequence alignments were manually edited to remove ambiguous positions and gaps. Phylogenetic trees were constructed by the neighbor-joining method [19] using the Kimura two-parameter model [20] in MEGA ver.6.0 [21]. Branch reliability was assessed using 1 000 bootstrap replicates [22]. DNA extraction, PCR amplification, DNA sequencing, and molecular phylogenetic analyses were performed at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan).

2.5. Scanning electron microscopy (SEM)

Morphological differences between the fungal isolate and the type strain *P. lilacinum* IFM 47467^T cultivated on PDA plates at 25 °C for 14 days were evaluated using an S-3400N scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

2.6. PHMB susceptibility testing

Spore suspensions of *P. lilacinum* strains were prepared from 2-week-old cultures by gently scraping the mycelial surfaces with sterile cutters, suspending the scrapings in 0.05% (w/v) Tween-80, and filtering the solutions through sterile gauze to collect the spores. The spores were counted using a hemocytometer, and homogenous suspensions of 10⁶ spores/mL were prepared. Then, 200 µL of 10⁶ spores/mL suspension was mixed with 20 mL of fresh 20% PHMB aqueous solution and incubated at 25 °C. At 3, 7, 14, and 28 days, the numbers of live spores were determined by diluting the suspensions in lecithin and polysorbate 80 solutions (Nihon Pharmaceutical Co., LTD, Tokyo, Japan) as neutralizers and spreading on PDA plates. The plates were incubated at 22.5 °C for 6 days, and colony-forming units (CFU) were counted. The detection limit was 2.0 log CFU/mL.

2.7. MIC testing

Spore suspensions of *P. lilacinum* strains were prepared from 2-week-old cultures by gently scraping the mycelial surfaces with sterile cutters, suspending the scrapings in 0.05% (w/v) Tween-80, and filtering the solutions through sterile gauze to collect the spores. The spores were counted using a hemocytometer, and homogenous suspensions of 10³⁻⁴ spores/mL were prepared.

The MICs of PHMB and four antimicrobial agents, namely methylparaben (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), phenoxyethanol (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), benzisothiazolin-3-one (Lonza, Basel, Switzerland), and benzalkonium chloride (Kao Co., Tokyo, Japan) against *P. lilacinum* strains and other fungi were determined using the broth dilution method [23], with minor modifications. Briefly, 0.1 mL of spore suspension containing 10^3 spores/mL and 0.1 mL 5-fold diluted potato dextrose broth containing various concentrations of antimicrobial agents were mixed well in 96-well microtiter plates (AGC Techno Glass CO., LTD., Shizuoka, Japan). The final concentrations were 0.010–10%, 0.0002–0.1%, 0.0021–1.1%, 0.00016–0.17%, and 0.00024–0.25% (w/v) for PHMB, methylparaben, phenoxyethanol, benzisothiazolin-3-one, and benzalkonium chloride, respectively, in serial two-fold dilutions. Duplicate mixtures were incubated at 25 °C for 3 days to determine the MICs.

The MICs of seven antifungal agents including micafungin, amphotericin B, 5-flucytosine, fluconazole, itraconazole, voriconazole, and miconazole against the type and resistant strains were determined by the microdilution method for filamentous fungi as proposed by the Clinical and Laboratory Standards Institute (document M38-A2 [24]). Spore suspensions of 10^4 spores/mL were prepared in RPMI 1640 broth and transferred to microtiter plates of antifungal sensitivity kit (Dry Plate; Eiken Chemicals Co., Ltd., Tokyo, Japan), which were incubated at 35 °C. After 48 h, growth inhibition was examined visually.

2.8. Genome sequencing, assembly, and annotation

The genomic DNA of the fungal isolate was extracted from a one-day-old culture using phenol-chloroform extraction and Nucleobond AXG column (TaKaRa) with Nucleobond buffer set III (TaKaRa). Genome sequencing was performed on a Pacific Biosciences RS II (Pacific Biosciences, Menlo Park, CA, USA) using libraries prepared with the SMRT- bell template prep kit 1.0 (Pacific Biosciences). A draft genome of the fungal isolate was assembled using SMRT Analysis 2.3 (Pacific Biosciences). The sequencing runs and assembly of the libraries were conducted by TaKaRa Bio (Mie, Japan).

Genome annotation was performed by using the Funannotate (v.1.7.4) pipeline [25]. The soft-masked genome by RepeatMasker (v.4.0.7) [26] and RepeatModeler (v.1.0.11) [27] was used for generating an *ab initio* gene model with the options `-busco_seed_species "fusarium_graminearum"` and `-busco_db "sordariomycetes"`. The completeness of the assembly and gene prediction was assessed using BUSCO v.4.0.5 with 3817 *Sordariomycetes* ortholog genes [28].

3. Results

3.1. Isolation of fungi from 20% aqueous solution of PHMB

White floating masses in a 20% aqueous solution of PHMB were collected for identification (Fig. 1A). The masses were first observed under a light microscope, which revealed the presence of fungal mycelial networks and spores (Fig. 1B). Pieces of mycelial structures were inoculated on PDA plates and incubated at 22.5 °C for 6 days. After cultivation, numerous brownish colonies were formed on all PDA plates (Fig. 1C).

3.2. Identification of fungal isolates

Among numerous brownish colonies isolated on PDA plates, five colonies were randomly picked for microbial identification based on ITS sequencing. The ITS sequences of the isolates were identical, and phylogenetic analysis revealed that they were highly similar (98.1–100%) to that of *P. lilacinum* (Fig. 2). Based on their morphology and ITS-based phylogeny, the isolates were all identified as *P. lilacinum*. One of the five strains, termed IFM 63780, was used in subsequent experiments as a representative PHMB-resistant fungal strain.

3.3. Morphology of the type and resistant strain

The morphological characteristics of the PHMB-resistant strain IFM 63780 were compared with those of the type strain IFM 47467^T. IFM 63780 colonies on PDA were slightly wrinkled and brownish like the type strain, and their growth was slower than that of the type strain (Fig. 3). Based on SEM, the morphology of conidiospore and hyphal formation of IFM 63780 were similar to those of IFM 47467^T (Fig. 4). These results indicated no significant difference in morphology and growth between the type strain IFM 47467^T and the resistant strain IFM 63780.

3.4. PHMB susceptibility of *P. lilacinum* strains

The PHMB susceptibility of seven cultured *P. lilacinum* strains and the resistant strain IFM 63780 was investigated by counting live spores after inoculating spore suspensions in freshly prepared 20% aqueous solution of PHMB. *P. lilacinum* IFM 47467^T spores were killed within 3 days, and the live spore counts for all other strains were decreased below the detection limit in 14 to 28 days (Table 2). In contrast, the live spore count of IFM 63780 did not decrease over 28 days, and white floating masses were observed within 7 days after inoculation and were confirmed to consist of mycelia by light microscopy. These results indicated that PHMB susceptibility of IFM 63780 is extremely low and that this strain can proliferate in a highly concentrated PHMB solution. A similar proliferative potential of IFM 63780 in a 20% aqueous solution of PHMB was observed after the 5th passage on PDA (data not shown), indicating strong and stable PHMB resistance of the isolate.

Table 2
Purpureocillium lilacinum survival (log CFU/mL) in 20% aqueous solution of PHMB

Strains	0 day	3 days	7 days	14 days	28 days
IFM 63780	4.0	4.0	> 4.0	> 4.0	> 4.0
IFM 47467 ^T	4.0	< 2.0	< 2.0	< 2.0	< 2.0
NBRC 5752	4.0	3.9	3.5	2.6	< 2.0
NBRC 31847	4.0	4.0	4.0	3.5	< 2.0
NBRC 31914	4.0	3.4	3.3	< 2.0	< 2.0
NBRC 31970	4.0	3.5	2.8	< 2.0	< 2.0
NBRC 32861	4.0	4.0	3.9	3.4	< 2.0
NBRC 103232	4.0	3.7	3.0	2.3	< 2.0

CFU, colony-forming units; PHMB, polyhexamethylene biguanide hydrochloride

3.5. Comparison of MICs

The MICs of PHMB, four other major antimicrobial agents, and seven antifungal agents against IFM 63780 in comparison with IFM 47467^T determined by the broth dilution method are shown in Tables 3 and 4, respectively. The MIC of PHMB against IFM 63780 was 50 000 µg/mL, which was approximately four-fold the MIC for IFM 47467^T (Table 3). Unexpectedly, the MICs of methylparaben, phenoxyethanol, benzisothiazolin-3-one, and benzalkonium chloride against IFM 63780 were lower than or equal to those against IFM 47467^T. A similar trend was observed for the antifungal agents, micafungin, amphotericin B, 5-flucytosine, fluconazole, itraconazole, voriconazole, and miconazole (Table 4). These results indicated that IFM 63780 is less susceptible than IFM 47467^T only to PHMB. The MICs of PHMB against four other pathogenic filamentous fungi and two yeasts were below 400 µg/mL and were consistently low (Table 5). Together, these results showed that *P. lilacinum* is relatively resistant to PHMB, with the isolate from this study being extremely resistant.

Table 3

MICs of PHMB and other antimicrobial agents against IFM 63780 and IFM 47467^T

Antimicrobial agent	MIC (µg/mL)	
	IFM 63780 (10 ³ spores/mL)	IFM 47467 ^T (10 ³ spores/mL)
PHMB	50 000	13 000
Methylparaben	250	500
Phenoxyethanol	2 800	5 500
Benzisothiazolin-3-one	< 1.6	3.2
Benzalkonium chloride	20	20
MIC, minimum inhibitory concentration; PHMB, polyhexamethylene biguanide hydrochloride		

Table 4

MICs of antifungal agents against IFM 63780 and IFM 47467^T

Antifungal agent	MIC (µg/mL)	
	IFM 63780 (10 ⁴ spores/mL)	IFM 47467 ^T (10 ⁴ spores/mL)
Micafungin (MEC)	< 0.015	0.03
Amphotericin B	> 16	> 16
5-Flucytosine	> 64	> 64
Fluconazole	16	16
Itraconazole	2.0	2.0
Voriconazole	0.25	0.25
Miconazole	2.0	2.0
MIC, minimum inhibitory concentration; MEC, minimum effective concentration		

Table 5
MICs of PHMB against other pathogenic filamentous fungi and yeasts

Inoculum (10 ³ spores/mL)	MIC (µg/mL)
<i>Aspergillus fumigatus</i> IFM 54229	< 400
<i>Aspergillus niger</i> IFM 62238	< 400
<i>Fusarium solani</i> IFM 62065	< 400
<i>Penicillium citrinum</i> IFM 62178	< 400
<i>Candida albicans</i> IFM 40009	< 400
<i>Cryptococcus neoformans</i> IFM 46660	< 400
MIC, minimum inhibitory concentration; PHMB, polyhexamethylene biguanide hydrochloride	

3.6. Genome features of the PHMB-resistant strain

We obtained a high-quality draft genome of IFM 63780 strain, comprising 11 scaffolds with a total length of 37 957 381 bp, an N_{50} value of 4 556 994 bases, and a GC content of 58.5%. The completeness of the draft genome was 99.8% with BUSCO. We predicted a total of 10 540 protein-coding genes. We found no gene homologous to the NCW2 gene that is responsible for cell tolerance to PHMB in yeasts after searching for genes previously reported to be involved in PHMB tolerance in *Saccharomyces cerevisiae* [13, 14].

4. Discussion

Purpureocillium lilacinum, formerly known as *Paecilomyces lilacinus*, is a ubiquitous and saprobic filamentous fungus commonly isolated from soil, decaying vegetation, insects, and nematodes [29]. *P. lilacinum* is also a pathogen and causes hyalohyphomycosis in humans [30], and reportedly produces paecilotoxin (leucinostatin) [31]. In this study, we successfully isolated the fungi from a highly concentrated (20%) PHMB aqueous solution and identified the PHMB-resistant isolates as *P. lilacinum*. Some strains of *P. lilacinum* can reportedly survive at a 20% concentration of PHMB. Previously, Huang *et al.* reported 100 µg/mL PHMB-tolerant clinical isolates of *Acanthamoeba castellanii* [32]. However, resistance to the extremely high PHMB concentration of 20%, which is 500–10 000-fold higher than that of industrially recommended concentrations, has not been reported to date. Such highly PHMB-resistant *P. lilacinum* strains can spoil and contaminate numerous PHMB-containing products, such as medical supplies, cosmetics, and household products, as well as production lines and public places (e.g., pools and bathing houses) where PHMB is used for decontamination. PHMB is also a preservative in solutions for contact lenses; thus, the resistant strains increase the risk of infections, particularly endophthalmitis [29], keratitis [33], and skin infection [34]. Nevertheless, our findings have considerable implications for microbial control. Indeed, our characterization of this pathogenic, highly drug-resistant fungus could be

valuable for manufacturers and consumers of PHMB-related products, aid in developing approaches to contain/treat contaminations, and prevent further emergence of drug resistance in fungi.

With regard to the microbiological profile of the new isolate, IFM 63780 could even proliferate in a 20% aqueous solution of PHMB, indicating that PHMB has no fungistatic or fungicidal effect on this strain, which could be utilizing PHMB as a sole carbon source. Our study revealed no significant difference in terms of morphology and growth between the type strain IFM 47467^T and the resistant strain IFM 63780. Moreover, no unusual cell structures such as thick-walled chlamydospores or hyphae, which are responsible for heat resistance of *Paecilomyces variotii* [35]—a typical heat-resistant fungus, were observed. The high resistance of IFM 63780 may be related to yet unidentified molecular mechanisms other than structural rearrangements of the cell membrane.

MICs of PHMB for IFM 63780 were found to be significantly higher, even when using average concentrations of 20–400 µg/mL. As PHMB is a cationic polymer, we expected strain IFM 63780 to be highly resistant to cationic agents; however, the MIC of benzalkonium chloride for IFM 63780 was identical to that for IFM 47467^T. *P. lilacinus* is known to be sensitive to voriconazole, but resistant to some antifungal agents, including amphotericin B [36], and the antifungal susceptibility test results for IFM 63780 were consistent with previous findings. IFM 63780 showed particularly high resistance, specifically to PHMB. Multidrug resistance refers to simultaneous resistance to unrelated drugs [37]. It has been recommended that combinations of multiple antimicrobial agents should be used to prevent the emergence of resistance to a single antimicrobial agent. Therefore, the combined antimicrobial spectrum would be extended, and the required concentration would decrease [38, 39]. With regard to the PHMB-resistant strain isolated in this study, multidrug resistance was not clearly observed; however, the possibility of acquiring multidrug resistance is conceivable based on previously reported cases.

IFM 63780 genome size, GC content, and gene count are almost identical to those of other *P. lilacinus* strains, such as PLFJ-1 (38.5 Mb, 58.3%, 11 763 genes, respectively) [40] and 36 - 1 (37.6 Mb, 58.4%, 13 150 genes, respectively) [41]. Although we expected a similar resistance mechanism and intended to identify a gene similar to that previously reported in yeast, no gene homologous to the NCW2 gene of *Saccharomyces cerevisiae* was found in the genome of IFM 63780. This result suggests that IFM 63780 acquired and developed the PHMB resistance through a mechanism that differs from that of yeasts and potentially involves PHMB resistance-related genes that are unique to filamentous fungi. The availability of the IFM 63780 genome sequence will contribute to our understanding of the molecular mechanisms underlying the biology of *P. lilacinus* and other fungi.

Our results justify efforts to increase manufacturer and consumer awareness regarding the use of PHMB. Additional studies are required to elucidate the resistance gene(s) and mechanism(s) of PHMB in the genus *Purpureocillium* and devise strategies to prevent the emergence of such highly drug-resistant fungi.

5. Conclusion

We identified a fungus contaminating a 20% aqueous solution of PHMB as *P. lilacinum*. The resistance level of the isolate, IFM 63780, was markedly higher than that of previously reported PHMB-resistant microorganisms. Moreover, the strain was found to be specifically resistant to PHMB among all antimicrobial agents tested in this study; however, its genome possessed no gene homologous to the PHMB resistance-related gene in yeasts. Although these findings indicate considerable medical safety concerns and severe public health risk, they highlight the presence of a novel drug resistance mechanism in the genus *Purpureocillium*.

Abbreviations

PHMB: polyhexamethylene biguanide hydrochloride, MIC: minimum inhibitory concentration, ITS: internal transcribed spacer, CFU: colony-forming units, PDA: potato dextrose agar

Declarations

Ethics approval and consent to participate

Not applicable

Adherence to national and international regulations

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Not applicable

Authors' contributions

TY performed the isolation and identification of fungi from a 20% aqueous solution of PHMB, PHMB susceptibility, and MIC testing, and was a major contributor in drafting the manuscript. HT performed the genome assembly and annotation and analyzed the genome data. YA investigated the microbiological

characteristics of the isolate and tested the MICs on PHMB. YK extracted genomic DNA and performed genome sequencing. KH provided the method of identification of the isolate and drafted part of the discussion and references. NS interpreted test data and contributed to the direction of research. SN participated in the general discussion on the research. TY summarized the study data and investigated the morphological characteristics of the isolate. All authors read and approved the final manuscript.

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Not applicable

References

- [1] Chadeau E, Brunon C, Degraeve P, Léonard D, Grossiord C, Bessueille F, Cottaz A, Renaud F, Ferreira I, Darroux C, Simon F (2012) Evaluation of antimicrobial activity of a polyhexamethylene biguanide-coated textile by monitoring both bacterial growth (iso 20743/2005 standard) and viability (live/dead baclight kit). *Journal of Food Safety* 32(2):141-151. <https://doi.org/10.1111/j.1745-4565.2011.00361.x>
- [2] Messick CR, Pendland SL, Moshirfar M, Fiscella RG, Losnedahl KJ, Schriever CA, Schreckenberger PC (1999) In-vitro activity of polyhexamethylene biguanide (PHMB) against fungal isolates associated with infective keratitis. *Journal of Antimicrobial Chemotherapy* 44(2):297-298. <https://doi.org/10.1093/jac/44.2.297>
- [3] Feng L, Wu F, Li J, Jiang Y, Duan X (2011) Antifungal activities of polyhexamethylene biguanide and polyhexamethylene guanide against the citrus sour rot pathogen *Geotrichum citri-aurantii* in vitro and in vivo. *Postharvest Biology and Technology* 61(2-3):160-164. <https://doi.org/10.1016/j.postharvbio.2011.03.002>
- [4] Valluri S, Fleming TP, Laycock KA, Tarle IS, Goldberg MA, Garcia-Ferrer FJ, Essary LR, Pepose JS (1997) In vitro and in vivo effects of polyhexamethylene biguanide against herpes simplex virus infection. *Cornea* 16(5):556-559.
- [5] Thomson JE, Cox NA, Bailey JS, Islam MN (1981) Minimizing salmonella contamination on broiler carcasses with poly (hexamethylenebiguanide hydrochloride). *Journal of Food Protection* 44(6):440-441. <https://doi.org/10.4315/0362-028X-44.6.440>
- [6] Mashat BH (2016) Polyhexamethylene biguanide hydrochloride: features and applications. *British Journal of Environmental Science* 4(1):49-55. ISSN 2055-0219(Print), ISSN 2055-0227(online)
- [7] Hubner NO, Kramer A (2010) Review on the efficacy, safety and clinical applications of polihexanide, a modern wound antiseptic. *Skin Pharmacology and Physiology* 23(suppl 1);17-27. <https://doi.org/10.1159/000318264>

- [8] Arch Chemicals, Inc., Norwalk, CT, USA, VANTOCIL™ IB technical information bulletin, http://www.excelind.co.in/Excel_Chemical/download/Vantocil_IB_Tech.pdf; 2005. Accessed 16 Dec 2019.
- [9] Makela PM, Korkeala HJ, Sand EK (1991) Effectiveness of commercial germicide products against the ropy slime-producing lactic acid bacteria. *Journal of Food Protection* 54(8):632-636. <https://doi.org/10.4315/0362-028X-54.8.632>
- [10] Henly EL, Dowling JAR, Maingay JB, Lacey MM, Smith TJ, Forbes S (2019) Biocide exposure induces changes in susceptibility, pathogenicity, and biofilm formation in uropathogenic *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 63(3):e01892. <https://doi.org/10.1128/AAC.01892-18>
- [11] Allen MJ, White GF, Morby AP (2006) The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide. *Microbiology* 152(4):989-1000. <https://doi.org/10.1099/mic.0.28643-0>
- [12] Elsztein C, de Lucena RM, de Morais MA Jr (2011) The resistance of the yeast *Saccharomyces cerevisiae* to the biocide polyhexamethylene biguanide: involvement of cell wall integrity pathway and emerging role for YAP1. *BMC Molecular Biology* 12:38. <https://doi.org/10.1186/1471-2199-12-38>
- [13] Elsztein C, de Lima RD, de Barros Pita W, de Morais MA (2016) *NCW2*, a gene involved in the tolerance to polyhexamethylene biguanide (PHMB), may help in the organisation of β -1,3-glucan structure of *Saccharomyces cerevisiae* cell wall. *Current Microbiology* 73:341-345. <https://doi.org/10.1007/s00284-016-1067-z>
- [14] Queiroz MG, Elsztein C, de Morais MA Jr (2020) The effects of the Ncw2 protein of *Saccharomyces cerevisiae* on the positioning of chitin in response to cell wall damage. *Antonie van Leeuwenhoek*. 113(2):265-277. <https://doi.org/10.1007/s10482-019-01335-y>
- [15] Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* 3(2):208-218. [https://doi.org/10.1016/S0022-2836\(61\)80047-8](https://doi.org/10.1016/S0022-2836(61)80047-8)
- [16] White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). *PCR protocols, a guide to methods and applications*. Academic Press, San Diego, p 315-322.
- [17] O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor, JW (eds). *The fungal holomorph, mitotic, meiotic and pleomorphic speciation in fungal systematic*. CAB International, Wallingford, p 225-233.
- [18] Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22(22):4673-4680. <https://doi.org/10.1093/nar/22.22.4673>

- [19] Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4):406-425.
<https://doi.org/10.1093/oxfordjournals.molbev.a040454>
- [20] Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16(2):111-120.
<https://doi.org/10.1007/bf01731581>
- [21] Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30(12):2725-2729.
<https://doi.org/10.1093/molbev/mst197>
- [22] Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39(4):783-791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
- [23] Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols* 3(2):163-175.
<https://doi.org/10.1038/nprot.2007.521>
- [24] Clinical and Laboratory Standards Institute Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard—2nd ed, CLSI document M38-A2, 2008. Clinical and Laboratory Standards Institute, Wayne, PA.
- [25] Palmer J (2016) Funannotate: pipeline for genome annotation.
<https://funannotate.readthedocs.io/en/latest/index.html>. Accessed 2 Mar 2020.
- [26] Smit AFA, Hubley R, Green P (2018) RepeatMasker Open-4.0. <http://www.repeatmasker.org>. Accessed 2 Mar 2020.
- [27] Smit AFA, Hubley R (2008) RepeatModeler Open-1.0. <http://www.repeatmasker.org>. Accessed 2 Mar 2020.
- [28] Seppey M, Manni M, Zdobnov EM (2019) BUSCO: assessing genome assembly and annotation completeness. *Methods in Molecular Biology*. 1962:227-245. https://doi:10.1007/978-1-4939-9173-0_14
- [29] Guo LN, Wang H, Hsueh PR, Meis JF, Chen H, Xu YC (2019) Endophthalmitis caused by *Purpureocillium lilacinum*. *Journal of Microbiology, Immunology, and Infection* 52(1):170-171.
<https://doi.org/10.1016/j.jmii.2017.12.002>
- [30] Peixoto ML, Santos DO, Souza Ide C, Neri EC, Sequeira DC, De Luca PM, Borba Cde M (2014) Interaction of an opportunistic fungus *Purpureocillium lilacinum* with human macrophages and dendritic cells. *Revista da Sociedade Brasileira de Medicina Tropical* 47(5):613-617. <https://doi.org/10.1590/0037-8682-0105-2014>

- [31] Mikami Y, Yazawa K, Fukushima K, Arai T, Udagawa S, Samson RA (1989) Paecilotoxin production in clinical or terrestrial isolates of *Paecilomyces lilacinus* strains. *Mycopathologia* 108(3):195-199. <https://doi.org/10.1007/bf00436225>
- [32] Huang FC, Shih MH, Chang KF, Huang JM, Shin JW, Lin WC (2017) Characterizing clinical isolates of *Acanthamoeba castellanii* with high resistance to polyhexamethylene biguanide in Taiwan. *Journal of Microbiology, Immunology, and Infection* 50(5):570-577. <https://doi.org/10.1016/j.jmii.2015.10.011>
- [33] Almeida Oliveira M, Carmo A, Rosa A, Murta J (2019) Posaconazole in the treatment of refractory *Purpureocillium lilacinum* (former *Paecilomyces lilacinus*) keratitis: the salvation when nothing works. *BMJ Case Report* 12(4):e228645. <https://doi.org/10.1136/bcr-2018-228645>
- [34] Trinh SA, Angarone MP (2017) *Purpureocillium lilacinum* tattoo-related skin infection in a kidney transplant recipient. *Transplant Infectious Disease* 19(3):e12689. <https://doi.org/10.1111/tid.12689>
- [35] Piecková E, Samson R (2000) Heat resistance of *Paecilomyces variotii* in sauce and juice. *Journal of Industrial Microbiology and Biotechnology* 24(4):227-230. <https://doi.org/10.1038/sj.jim.2900794>
- [36] Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA (2011) *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. *FEMS Microbiology Letters* 321(2):141-149. <https://doi.org/10.1111/j.1574-6968.2011.02322.x>
- [37] Alekshun MN, Levy SB (2007) Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128(6):1037-1050. <https://doi.org/10.1016/j.cell.2007.03.004>
- [38] Ettayebi K, El Yamani J, Rossi-Hassani B (2000) Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. *FEMS Microbiology Letters* 183(1):191-195. <https://doi.org/10.1111/j.1574-6968.2000.tb08956.x>
- [39] Leekha S, Terrell CL, Edson, RS (2011) General principles of antimicrobial therapy. *Mayo Clinic Proceedings* 86(2):156-167. <https://doi.org/10.4065/mcp.2010.0639>
- [40] Gang W, Zhiguo L, Runmao L, Erfeng L, Zhenchuan M, Jian L, Yuhong Y, Wen-Bing Y, Bingyan X (2016) Biosynthesis of antibiotic leucinostatins in bio-control fungus *Purpureocillium lilacinum* and their inhibition on phytophthora revealed by genome mining. *Plos Pathogens*. 12(7):e1005685. <https://doi.org/10.1371/journal.ppat.1005685>
- [41] Jialian X, Shaojun L, Chenmi M, Xueqiong X, Deliang P, Gaofeng W, Yannong X (2016) Genome and transcriptome sequences reveal the specific parasitism of the nematophagous *Purpureocillium lilacinum* 36-1. *Frontiers in Microbiology*. 7:1084. <https://doi.org/10.3389/fmicb.2016.01084>

Figures

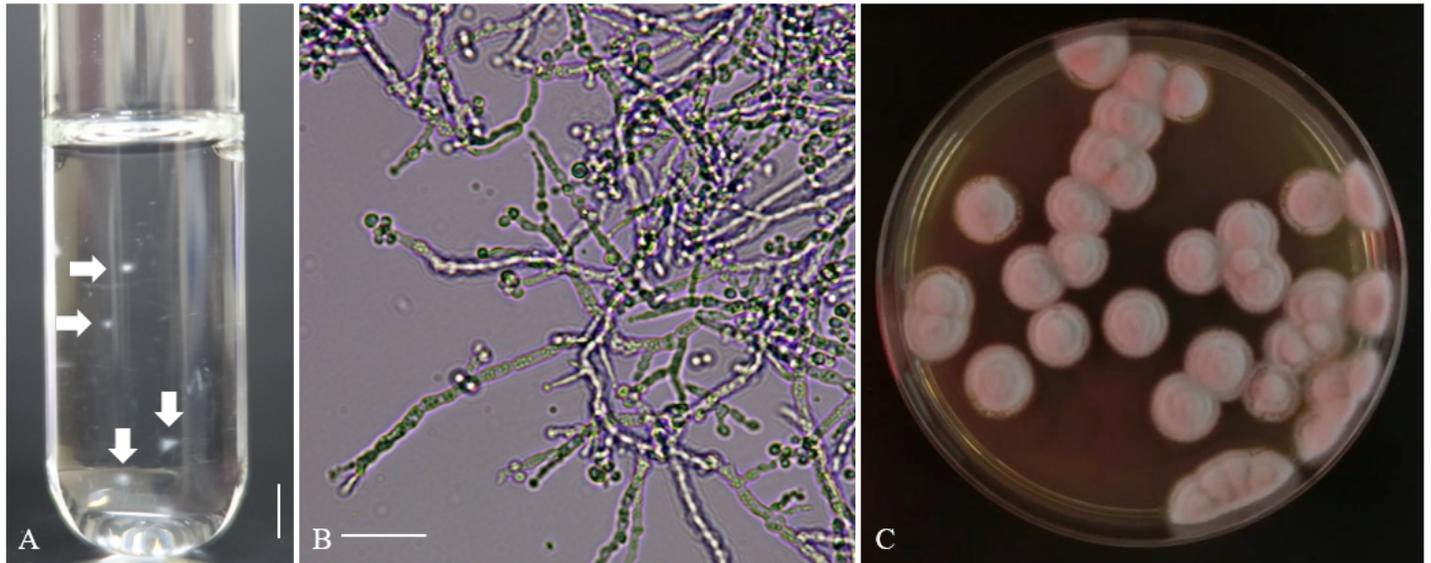


Figure 1

(A) White floating masses in a 20% aqueous solution of polyhexamethylene biguanide hydrochloride (PHMB). Scale bar = 5 mm. White arrows indicate the floating masses that were observed after leaving the PHMB in polyethylene containers to stand for more than 1 month. (B) Mycelia and spores of the white floating masses observed in a 20% solution of PHMB. Scale bar = 20 μm (light microscope). (C) Colonies formed from mycelium of the floating masses inoculated on potato dextrose agar at 22.5 $^{\circ}\text{C}$ for 6 days.

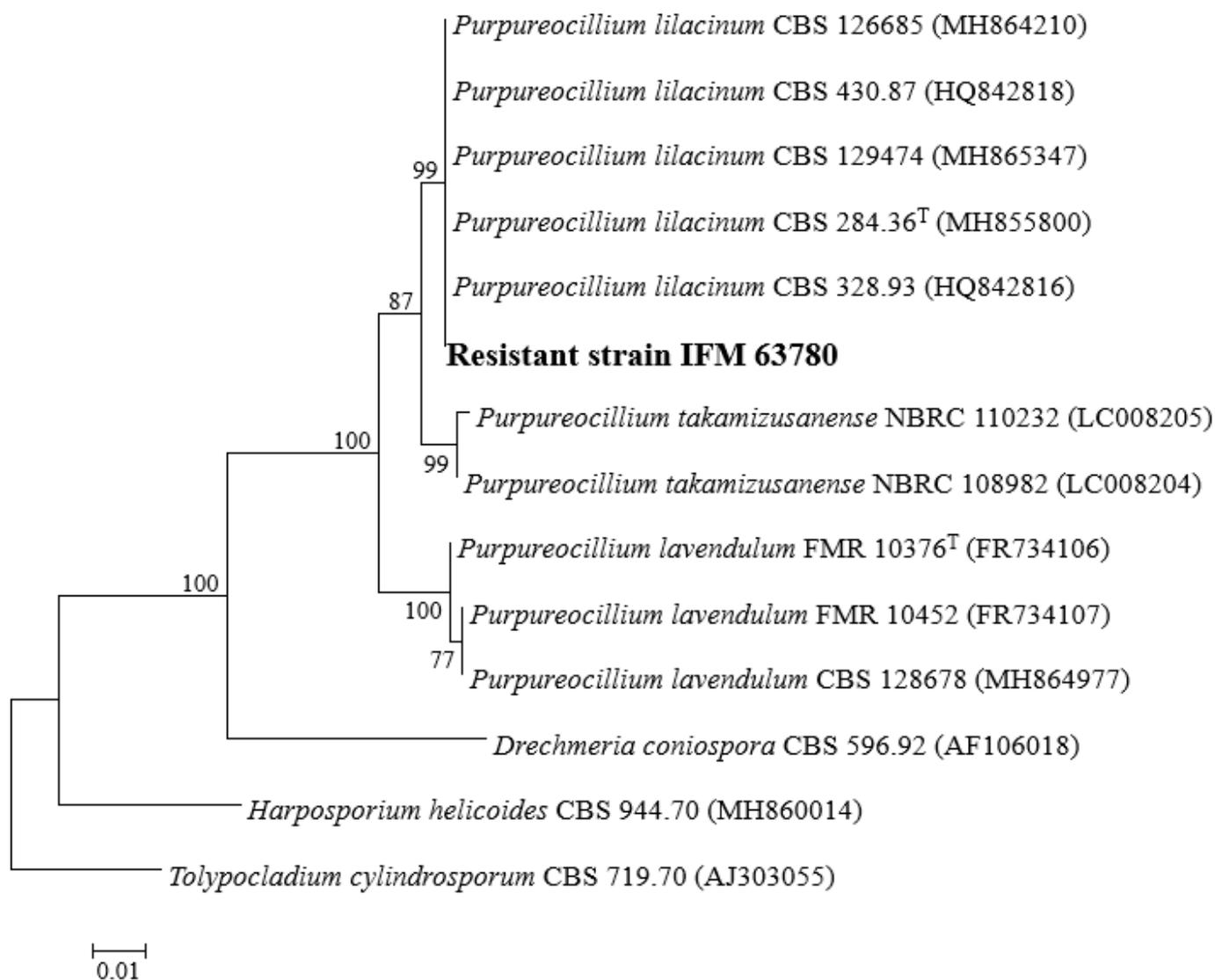


Figure 2

Neighbor-joining tree of the polyhexamethylene biguanide hydrochloride-resistant strain and its relatives based on ITS region sequences. Bootstrap values above 70% are indicated near each cluster; scale bar at the bottom left indicates the number of substitutions per site; T: ex-type strain of the species.

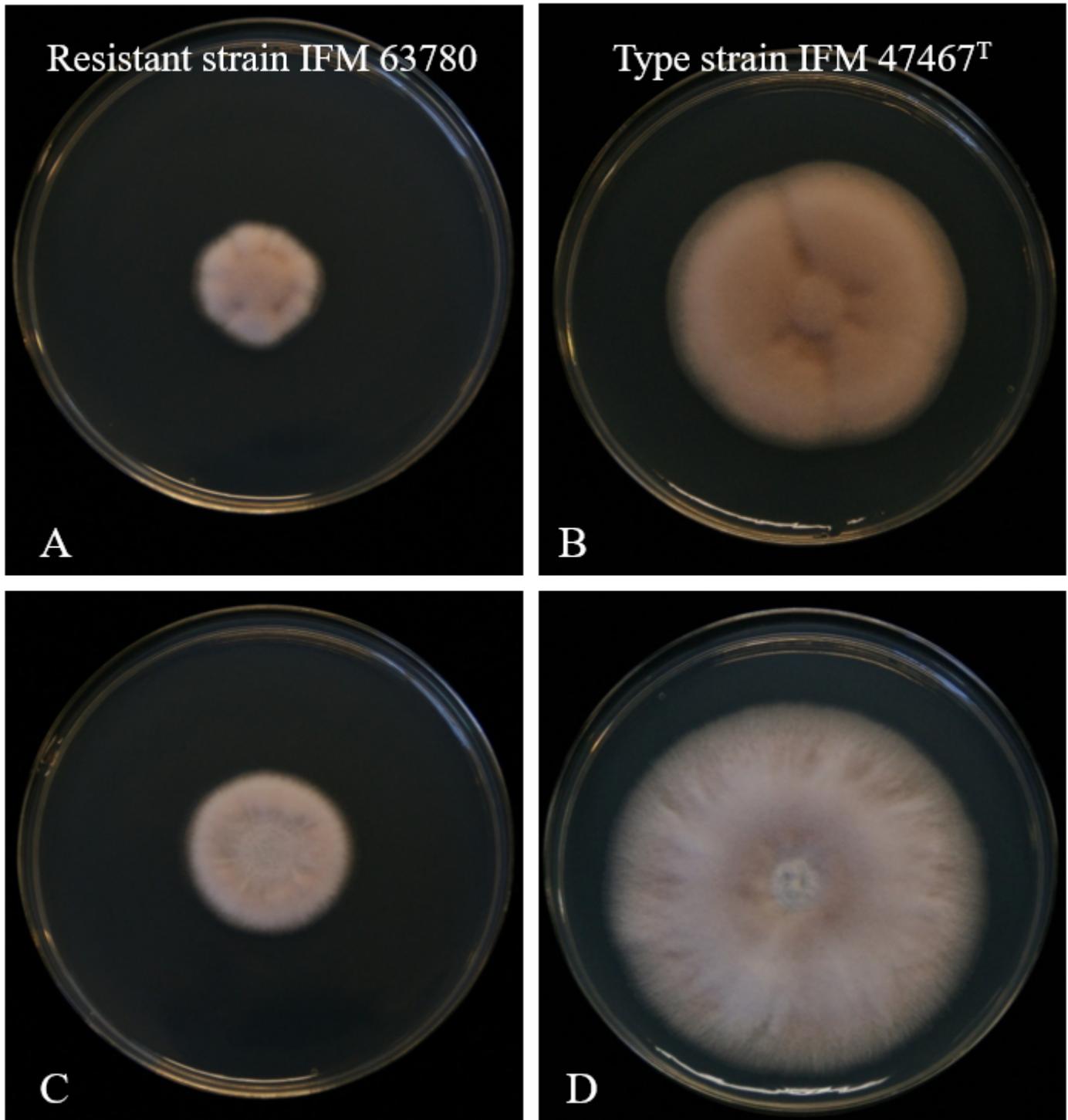


Figure 3

Purpureocillium lilacinum colonies on potato dextrose agar after incubation at 25 °C for 7 days (A, B) or 14 days (C, D). IFM 63780 (A, C) IFM 47467 T (B, D).

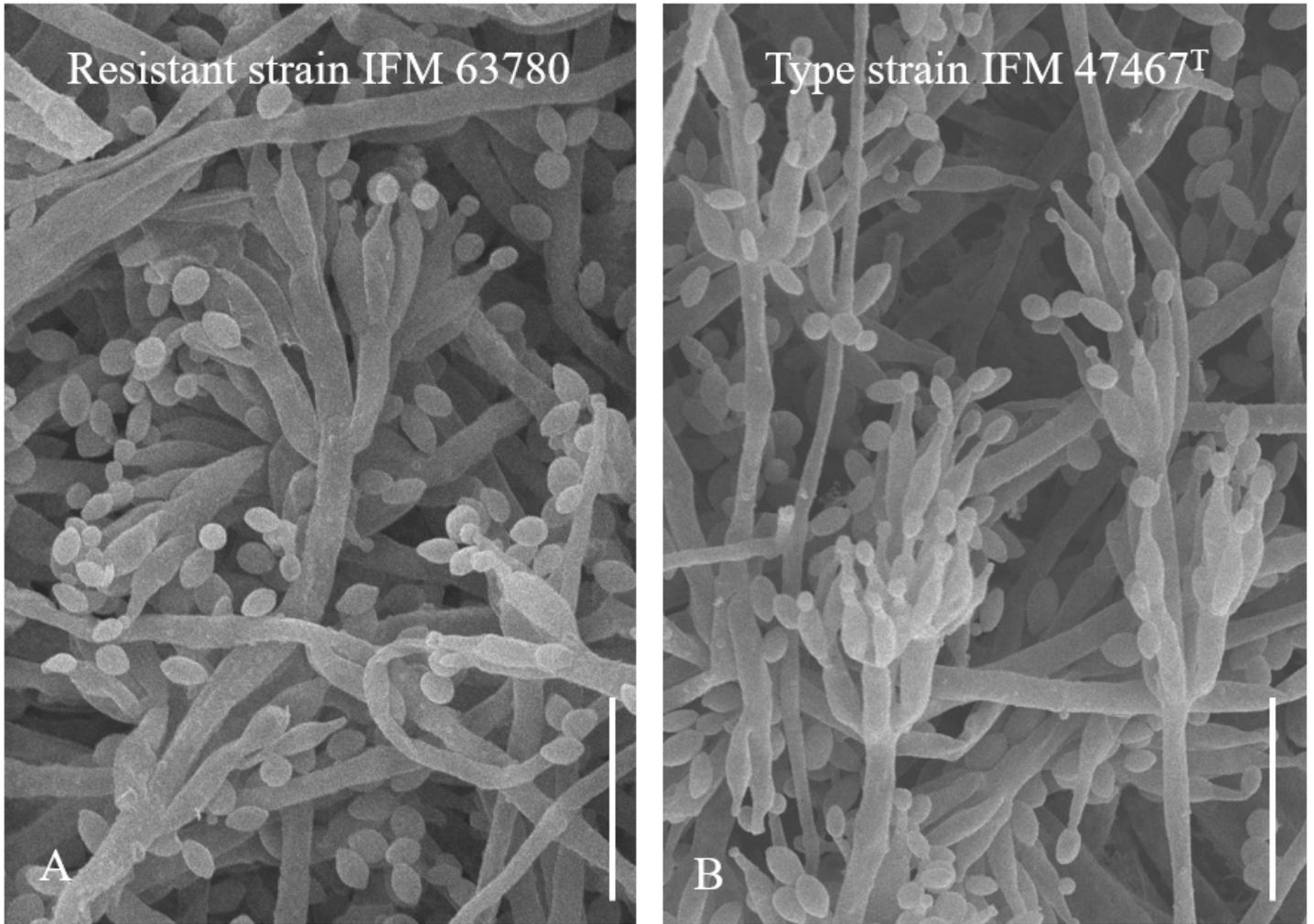


Figure 4

Scanning electron microscopy of *Purpureocillium lilacinum* grown on potato dextrose agar at 25 °C for 14 days. IFM 63780 (A), IFM 47467T (B). Scale bars = 10 μ m.