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ORIGINAL ARTICLE

Aspergillus mulundensis sp. nov., a new species for the fungus producing the antifungal echinocandin lipopeptides, mulundocandins

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The invalidly published name *Aspergillus sydowii* var. *mulundensis* was proposed for a strain of *Aspergillus* that produced new echinocandin metabolites designated as the mulundocadins. Reinvestigation of this strain (Y-30462 = DSMZ 5745) using phylogenetic, morphological, and metabolic data indicated that it is a distinct and novel species of *Aspergillus* sect. *Nidulantes*. The taxonomic novelty, *Aspergillus mulundensis*, is introduced for this historically important echinocandin-producing strain. The closely related *A. nidulans* FGSC A4 has one of the most extensively characterized secondary metabolomes of any filamentous fungus. Comparison of the full-genome sequences of DSMZ 5745 and FGSC A4 indicated that the two strains share 33 secondary metabolite biosynthetic gene clusters. These shared gene clusters represent ~45% of the total secondary metabolome of each strain, thus indicating a high level intraspecific divergence in terms of secondary metabolism.
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INTRODUCTION

Mulundocandin and deoxymulundocandin (Figure 1: 1, 2) are lipohexapeptides and potent antifungal antibiotics of the echinocandin class.[1–3] Biosynthetically, they are closely related to echinocandin B and C (Figure 1: 3, 4) but differ by the inclusion of serine instead of threonine in the fifth position of the hexapeptide core and by a 12-methyl myristoyl side chain instead of a lineolyl side chain. Mulundocandin and its deshydroxy C4 homotyrosine form have been investigated extensively as potential lead structures for the development of echinocandin-type antifungal drugs.[3–6] The structure elucidation of mulundocandin and its potent antifungal activity against *Candida albicans*, *Cercospora beticola* and *Microsporum gypseum* were described in 1987 from a strain of *Aspergillus* (Y-30462 = DSMZ 5745) isolated at Hoechst India Ltd located in Mulund district of Mumbai, India from a soil sample collected in Bangladesh.[1,2] In the original publication, the fungus was considered to be an unusual variant of *Aspergillus sydowii* because of the presence of abundant Hülle cells and was designated as *A. sydowii* var. *mulundensis*. However, the name was published as a *nomen nudum* because no type specimen was designated and no Latin diagnosis was provided. During the course of development of a transformation method for the mulundocandin-producing strain by gene inactivation with the hygromycin resistance gene, the internal transcribed spacer ribosomal DNA of a subculture derived from DSMZ 5745 was sequenced (AJ312221), and the sequence data indicated the fungus was more closely related to *A. nidulans* than to *A. sydowii*.[7]

Recently, the full spectrum of echinocandin-type biosynthetic gene clusters, including the gene clusters encoding the starting molecules for the antifungal drugs, caspofungin, micafungin and anidulifungin, was investigated to determine the evolutionary origins and relationships of the echinocandins and to better understand the chemical logic underpinning the biosynthesis of this class of potent antifungal metabolites.[8,9] Strain Y-30462 was purchased from the DSMZ patent collection (DSMZ 5745), and its genomic DNA was extracted and used for genomic sequencing to elucidate the mulundocandin biosynthetic gene cluster and to search for additional biosynthetic genes that might be associated with mulundocandin biosynthesis and regulation.[9] Sequencing the genome of DSMZ 5745 afforded us access to ribosomal and protein-encoding genes that could be used as phylogenetic markers. New sequence data and morphology confirmed that strain DSMZ 5745 was more closely allied to species in the *A. nidulans* group in *Aspergillus* sect. *Nidulantes* than to species in the sect. *Versicolores* (*A. sydowii*–*A. versicolor* group). Inclusion of strain DSMZ 5745 in sect. *Nidulantes* would be consistent with previous observations that multiple species and strains in sect. *Nidulantes* can produce echinocandins.[10,11]

The objective of this report was to clarify the name of the mulundocandin-producing strain and determine whether it was one of the known taxa in the species complex surrounding *A. nidulans* or whether it might represent a new species. The hypothesis was tested by probing current databases with ribosomal DNA sequences for this strain and by resampling available phylogenetic marker sequences to

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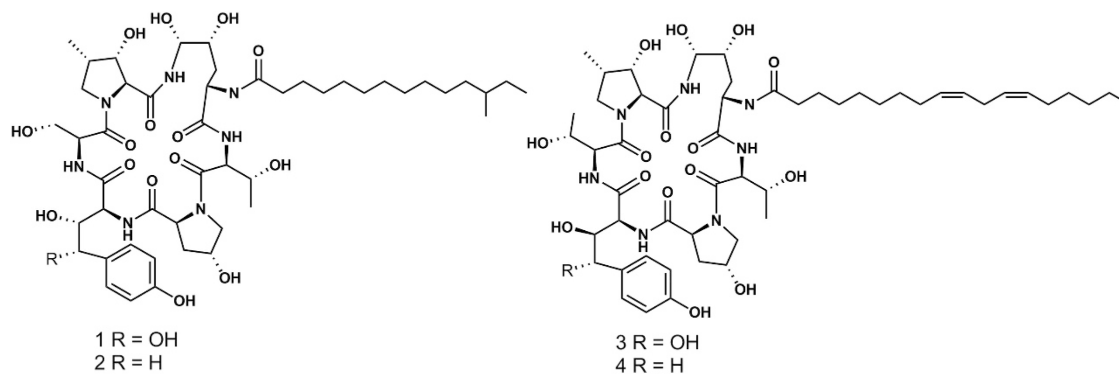

Figure 1 Chemical structures of *Aspergillus* echinocandins. Mundundocandin (1), Dexoxymulundocandin (2), Echinocandin B (3), Echinocandin C (4).

build multi-gene phylogenies. We also examined this question by evaluation of the strain's morphological characteristics and extrolite profiles. At last, the availability of a full-genome sequence for the mulundocandin-producing strain gave us an opportunity to supplement chemotaxonomic data with measurements of the potential divergence in secondary metabolism between two genome-sequenced species within Sect. *Nidulantes*. Therefore, we compared the genomic sequence of strain DSMZ 5745 with a strain of the central species of the *A. nidulans* complex, that of *A. nidulans* FGSC A4, probably the most studied secondary metabolome available from any fungus,.[12–15] These data provide one of the first examples of using secondary metabolite gene clusters from sequenced genomes to aid in delimiting a new species and lead to the conclusion that the mulundocandin-producing strain represents a previously unrecognized species in the *Aspergillus* sect. *Nidulantes*.

MATERIALS AND METHODS

Strain and morphological characterization

The sole strain of *A. sydowii* var. *mulundensis* Y-30462 deposited as DSMZ 5745[16] was purchased from the patent collection at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The strain was inoculated on Czapek yeast autolysate agar (CYA agar), malt extract agar (MEA) (Blakeslee formula, with Difco malt extract and peptone), MEA using the Oxoid formula (MEAOx), yeast extract sucrose agar, oat meal agar (from organic oat meal) and creatine sucrose agar and all were incubated at 25 °C.[17] Furthermore, the strain was incubated at 37 °C on CYA agar. Photomicrographs were taken from cultures grown on cornmeal agar at room temperature.

Molecular phylogenetic analysis

To clarify the phylogenetic status of *A. sydowii* var. *mulundensis* DSMZ 5745, we constructed a phylogenetic tree from a data set of the concatenated DNA fragments of the actin, calmodulin and β-tubulin genes that were resampled from a recent analysis of the *A. nidulans* species complex.[18] The actin gene of DSMZ 5745 was amplified using primer pairs ACT-512F and ACT-783R,[19] and other genes were extracted from the sequenced and annotated genome of DSMZ 5745.[9] These sequences have been accessioned to Genbank (KP985733, KP985734, KP985735). The concatenated gene sequences for each species were aligned with ClustalW implemented in MEGA 6.0,[20] and analyzed by the maximum likelihood method based on a K2+G+I model using MEGA 6.0. The data matrix and tree were accessioned to TreeBASE (www.treebase.org) as study 17905.

In addition to the sequences mentioned above, the nuclear ribosomal internal transcribed spacer and large subunit were amplified and sequenced and accessioned to GenBank (KP985732).

Extrolite analysis

For extrolite analysis, three agar plugs were taken from CYA, MEA, yeast extract sucrose, OAT and MEAOx and extracted using established methods.[21] The extracts were analyzed using ultra high pressure liquid chromatography-diode array detector (UHPLC-DAD) as described previously.[22]

Furthermore, we approximated the original fermentation conditions used for production of deoxymulundocandin to verify that DSMZ 5745 was still able to produce mulundocandins. The strain was cultured on malt-yeast extract agar at 25 °C for 7 days to produce conidia and mycelia. Agar cultures were cut into small pieces (0.5 cm^3), and 15 pieces were inoculated into 50 ml of SMYA medium (Bacto neopeptone 10 g, maltose 40 g, yeast extract 10 g, agar 4 g, deionized H_2O 1000 ml) in a 250-ml Erlenmeyer flask. This seed culture was incubated at 25 °C on a rotary shaker at 220 rpm for 3 days. A 10-ml aliquot of the seed medium was inoculated into 100 ml of production medium (NZ amine EKC (N4767 Sigma-Aldrich, St Louis, MO, USA) 3 g, Bacto peptone 3 g, glucose 10 g, soluble starch 24 g, yeast extract 5 g, $CaCO_3$ 4 g, $MnCl_2.4H_2O$ 0.5 mg, $ZnSO_4.7H_2O$ 0.22 mg, $CaCl_2$ 0.55 mg, $FeSO_4.7H_2O$ 0.5 mg, $CuSO_4.5H_2O$ 0.16 mg, $CoCl_2.6H_2O$ 0.16 mg, deionized H_2O 1000 ml) in each of two 500-ml Erlenmeyer flasks. Flasks were incubated at 25 °C on a rotary shaker at 220 rpm for 76 h.

A culture from one flask was vacuum-filtered to separate mycelium and culture broth. Each was extracted with 100 ml of ethyl acetate, respectively. In the second culture, 100 ml of ethyl acetate was added directly to extract the mycelium and broth simultaneously. The organic phase was evaporated to dryness and redissolved in methanol (MeOH) at 30 mg ml^{-1}. Then 10 μl of each dissolved extracts were injected for HPLC-DAD-mass spectrometry (HPLC-DAD-MS) analysis on a linear gradient of 10–90% acetonitrile in water (with 0.1% formic acid) for 28 min at a flow rate of 1 ml min^{-1} through an Agilent Zorbax Eclipse Plus C_{18} reverse phase column (4.6 × 150 mm, 5 μm).

Verification and structural elucidation of mulundocandins

After the LC-MS analysis, the crude extract (0.4 g) was fractionated with a reversed-phase C_{18} column (10–100% MeOH in H_2O over 25 min; 40 ml min^{-1}) coupled to a Grace Reveleris X2 flash chromatography system. Fractions (25 mg) eluted with 60 and 70% MeOH were combined and further purified by semi-preparative reverse phase HPLC (Agilent Zorbax SB-C_{18} column; 5 μm; 9.4 × 250 mm; 60% acetonitrile in H_2O over 15 min; 2 ml min^{-1}) to afford 1 (1.2 mg, t_R 8.37 min) and 2 (2.1 mg, t_R 10.23 min).

NMR data were collected on a Bruker 500 MHz NMR equipped with a 5-mm triple resonance cryoprobe at 298 K. Residual solvent signals were used as reference (CD_3OD: δ_H 3.31/δ_C 49.2). The high-resolution mass spectra for each compound were acquired with an Agilent 6520 Q-TOF system in the positive ionization mode. For Q-TOF/MS conditions, voltages of fragmentor and capillary were kept at 130 and 3500 V, respectively. Nitrogen was provided as the nebulizing and drying gas. Temperature of the drying gas was kept at 300 °C. The flow rate of the drying gas and the pressure of the nebulizer were 10 l min^{-1} and 25 psi, respectively. Full-scan spectra were acquired over a scan range of m/z 80–1500 at 1.03 spectra s^{-1}. The ^1H NMR and the

high-resolution mass data for mulundocandin (1) and deoxymulundocandin (2) were consistent with previous literature values.[1,3]

Comparison of secondary metabolomes of DSMZ 5745 and *A. nidulans* FGSC A4

The repeat-masked nucleotide scaffolds and predicted proteins of *Aspergillus mulundensis* DSMZ 5745 were analyzed with anti-SMASH,[23] employing the default parameters to identify the core secondary metabolite genes and to approximately delimit biosynthetic gene clusters. Previously determined core genes and gene clusters from *A. nidulans* were used as references sequences to basic local alignment search tool (BLAST) against the *A. mulundensis* genome to manually locate orthologous gene clusters.[12,24] Orthologous gene clusters between *A. mulundensis* and *A. nidulans* were aligned and illustrated using Easyfig (Supplementary Figure S1) to determine gene identity and microsynteny.[25]

RESULTS

Phylogenetic analysis

Basic local alignment search tool searches with internal transcribed spacer and large subunit sequences of DSMZ 5745 consistently retrieved high scoring similarities for various species of *Aspergillus* sect. *Nidulantes,* including *A. falconensis*, *A. similis*, *A. multicolor*, *A. omanensis*, A. *navahoensis* and *A. fruticans*; however, no particular species provided an unequivocal similarity match. Curiously, the closest match for an environmental DNA sequence using the internal transcribed spacer region was uncultured fungus clone 06CI5ID11 (KP243146, 96% identity), thus indicating the species is not commonly encountered during metagenomic pyrosequencing or cloning experiments. We attempted to identify strain DSMZ 5745 and associate it with a previously described species of *Aspergillus* sect. *Nidulantes*. We reconstructed the data matrix from a recent phylogenetic investigation of *Aspergillus* sect *Nidulantes*[18] using the concatenated actin, calmodulin and β-tubulin gene sequences and tested whether the corresponding sequences from DSMZ 5745 would cluster unambiguously with one of the known species. Tree topology resulting from maximum likelihood analysis closely tracked the previous analysis (Figure 2). Sequences for DSMZ 5745 were intercalated deeply in a basal branch of the tree, in a strongly supported, but isolated branch with *A. pluriseminatus* (Figure 2). Its location on this isolated node was consistent with the hypothesis that DSMZ 5745 was a previously unrecognized species. The relatively isolated position among the other species and strains in the tree was consistent with the chemical uniqueness of the strain based on extrolite and genomic analysis (Supplementary Table S1), and led to the conclusion that the strain represented a previously unrecognized species.

Taxonomy

Aspergillus mulundensis Bills and Frisvad *sp. nov.* MycoBank MB813062

≡ *Aspergillus sydowii* var. *mulundensis*, Journal of Antibiotics 40:275, 1987, *nomen nudum*

Typus: DSMZ 5745^{T} patent collection; IBT 33104^{T} (Figure 3a–i), ex soil, Bangladesh

Etymology: from Mulund, Mumbai, India, site of the former headquarters of Hoechst India Ltd, where the fungus was isolated and first recognized to produce a new echinocandin-type antibiotic, mulundocandin.

Diagnosis: *A. mulundensis* differs from *A. nidulans* by producing smaller penicillate heads, shorter conidiophore stipes, by growing more slowly on all media, especially at 37 °C, and by not producing ascomata. It differs from *A. sydowii* by having metulae only covering the upper 50% of the conidial heads, by having dark dull green conidia, and by not growing well on creatine sucrose agar. Hülle cells were not observed. The *A. mulundensis* genome harbors the mulundocandin gene cluster that is absent in *A. nidulans*, but it lacks genes for biosynthesis of sterigmatocystin and penicillin that are characteristic of *A. nidulans*.

Description: colonies on CYA. 1 week, 25 °C, 20–22 mm, poor sporulation, white mycelium and a yellow–brown reverse. Colonies on MEA, 1 week, 25 °C, 7–15 mm, no sporulation, white mycelium, and an orange–brown reverse, colonies on yeast extract sucrose agar 1 week, 25 °C, 27–35 mm, no sporulation, white mycelium, warm orange–brown reverse, colonies on oat meal weakly sporulating, whereas mycelium, cream-colored reverse. On CYA at 37 °C, colonies 3–9 mm, no sporulation and white mycelium and cream-colored reverse. Conidia *en masse* colored dark dull green to dull blue–green, and produced best on MEA and oat meal agars after 2 weeks of incubation. Conidial heads radiate and globose. On creatine sucrose very poor growth, 5–16 mm diameter, and no acid production. Fully developed Aspergilli biseriate with metulae covering the upper 50–60% of the head, vesicle small, globose to pear shaped, 8–20 μm, metulae 5–7 μm × 2–3 μm, cylindrical, each having 1–3 phialides that are short, 5–8 μm × 2.5–4 μm, with broad collula, stipes smooth- and thick-walled, uncolored to light brown, short, in some aspergilla 7–12 μm × 5–7 μm, others are longer 25–120 μm × 5–7.5 μm. Several single phialides and small penicillate heads are also produced, similar to those seen in *A. sydowii* and *A. asperescens*,[26,27] especially on aerial hyphae. In some conidial heads, phialides are intermixed with metulae and phialides. Conidia globose, delicately spinulose to verrucose, 2.5–4.5 μm diameter. Ascomata and sclerotia not observed, not even in cultures grown on autoclaved rehydrated wheat seeds in ambient light for 1 month. Hülle cells were not observed, but *fide* Roy *et al.*,[2] 'Hülle cells are abundant, thick-walled, colorless at first becoming purple with age, scattered unevenly, 18.7–31.2 × 25–37.5 μm'.

The type strain (Figure 3a–i) can produce azaphilone extrolites, but they are not falconensins produced by the related *Aspergillus falconensis*,[28–31] but rather are related to sclerotiorins with UV spectra identical to those in the extracts of *Penicillium sclerotiorum*. In addition, *A. mulundensis* produces extrolites with UV spectra suggesting karnatakafurans or similar dibenzofurans.[32,33] The putative dibenzofuran had a UV spectrum with absorptions at 230 nm (100%), 265 (45%), 287 (28%), 303 (17%), 314 (22%). Finally, this strain continued to produce mulundocandin and deoxymulundocandin when grown on a medium similar to the Hoechst production medium;[2,3,16] however, these extrolites were undetectable on the standardized agar media and extraction method used for extrolite characterization (Figure 4). As expected, the liquid fermentation extracts were strongly inhibitory to growth of *C. albicans* ATCC 10231 in an agar zone of inhibition assay (data not shown). Like the pneumocandins, mulundocandins were produced during exponential growth and were strongly associated with the mycelium and were not secreted in the culture broth (Figure 4).

The predicted secondary metabolome of the *A. mulundensis*

The mulundocandin gene cluster was recently delineated in *A. mulundensis*,[9] and it was pointed out this strain shared in common with *A. nidulans*, the gene clusters for the lipopentapeptide, emericellamide and a two-module non-ribosomal peptide synthetases (NRPS) *inp* gene cluster that is not yet linked to a known metabolite. Analysis with Anti-SMASH and BLAST searches with key polyketide synthase and NRPS domains revealed a rich repertoire of secondary metabolite biosynthetic genes including 33 polyketide synthases (PKS),

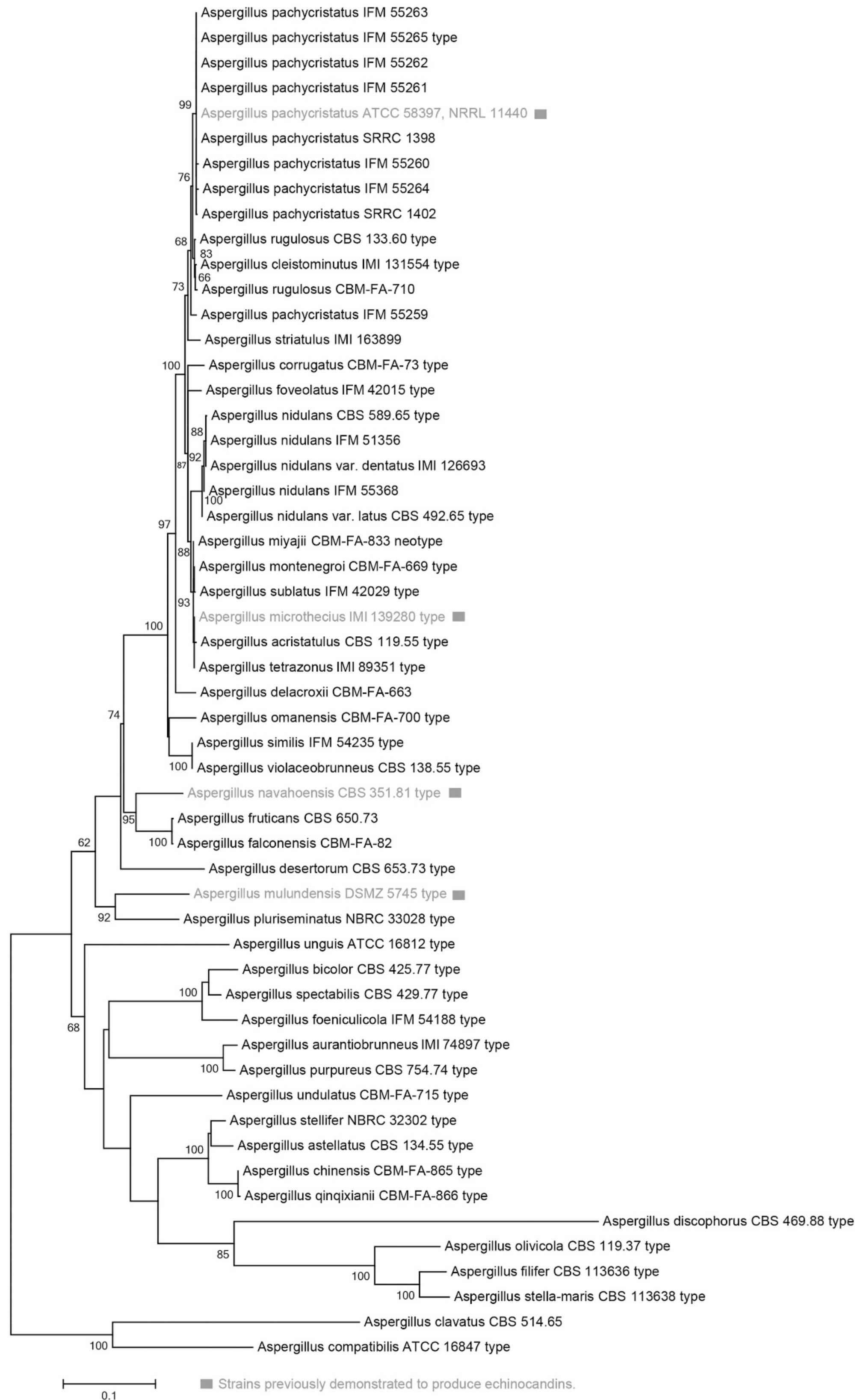

Figure 2 Maximum likelihood analysis of *Aspergillus mulundensis* DSMZ 5745 and selected fungi of *Aspergillus* sect. *Nidulantes* based on a combined three-gene data set including the DNA fragments of actin, calmodulin and β-tubulin genes. The evolutionary history was inferred by using the maximum likelihood method based on a K2+G+I model. Branch lengths are proportional to the number of genetic changes. Numbers at branch points represent bootstrap values >60% of 1000 bootstrap replications. The alignment data matrix consisted of 1795 characters and 54 strains. Strains in red with a red box have been verified to produce echinocandins. A full color version of this figure is available at The *Journal of Antibiotics* journal online.

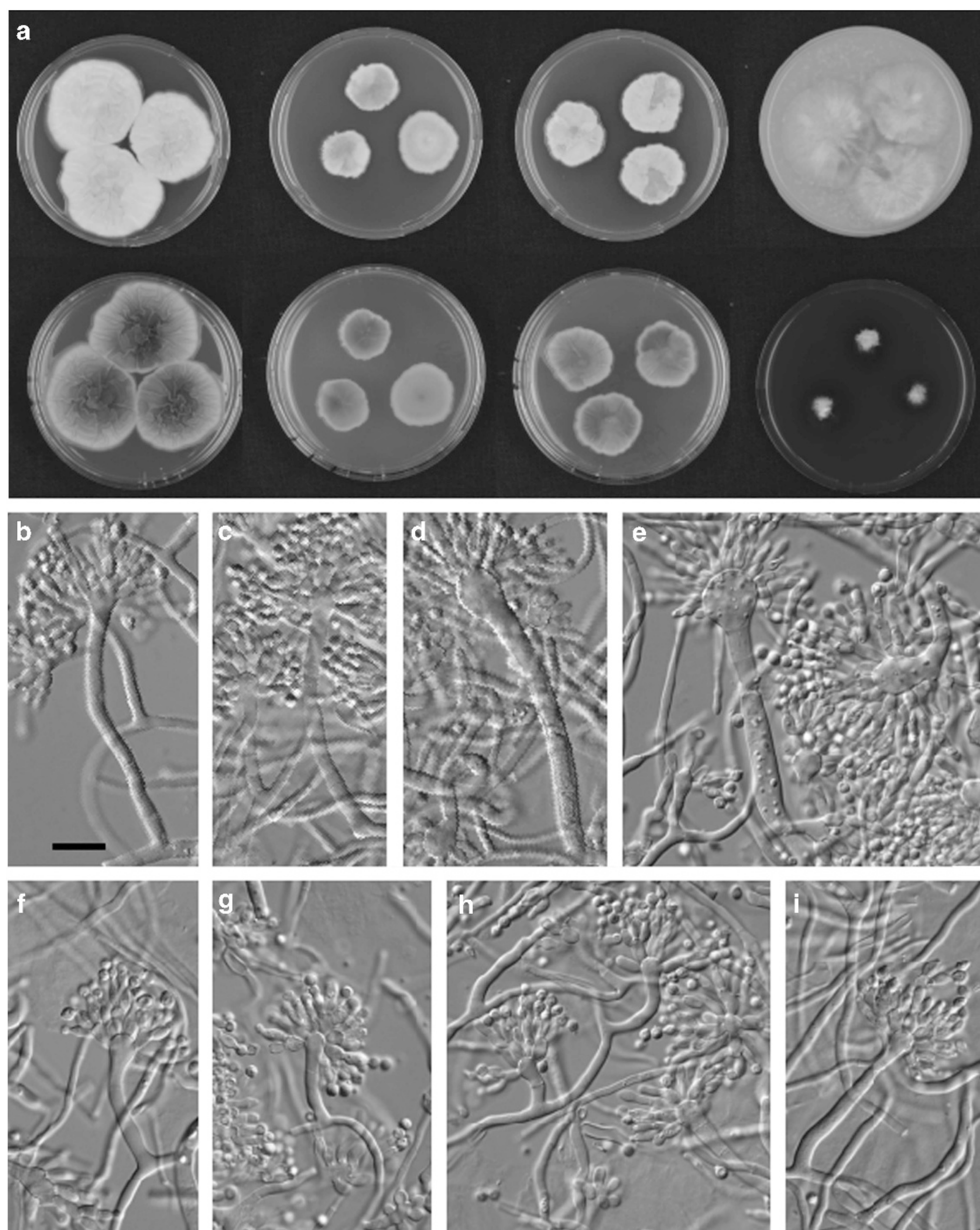

Figure 3 *Aspergillus mulundensis*, type strain DSMZ 5745 = IBT 33104. (**a**) Seven-day old colonies. Top row, left to right, YES, MEA, CYA, OA; Bottom row YES reverse, MEA reverse, CYA reverse, CREA. (**b–i**) Conidiophores and conidia. All photographed at the same scale, scale bar in B = 10 μm. A full color version of this figure is available at The *Journal of Antibiotics* journal online.

19 NRPS, 10 terpene synthases and 5 dimethylallyl tryptophan synthases dispersed among 73 gene clusters (Figure 5). This estimate is similar to the ~71 core genes and gene clusters identified in *A. nidulans* FGSC A4.[12] Secondary metabolite gene clusters previously identified in FGSC A4 were aligned to annotated scaffolds from DSMZ 5745 to verify that other secondary metabolite gene clusters not only shared significant sequence homology, but also were similar in microsynteny (Supplementary Figure 1). In total, 33 gene clusters were found to be highly orthologous (Figure 5) and to share significantly similar microsynteny (Supplementary Figure S1). Besides the presence of the mulundocandin and emericellamide gene clusters, an additional 9 of these 33 other gene clusters were highly similar to those in *A. niduluans* that have been linked to characterized compounds or compound families (Supplementary Figure S1). The analysis predicted that DSMZ 5745 should produce asperfuranones,[34] alternariol or related isocoumarins, the non-ribosomal peptide-terpenes nidulanin and fungisporin,[12,24] orsellinaldehyde, orsellinic acid and derivatives, asperthecin,[35] the siderophore ferricrocin[36] and a green conidial pigment encoded by *wA*. However, most of the shared gene clusters are not yet linked with a known metabolite.

DISCUSSION

Aspergillus mulundensis originated from a soil sample from an unspecified location in Bangladesh. Based on our investigation, *A.sydowii* var. *mulundensis* should be considered a homotypic synonym because DSMZ 5745 was derived from Hoechst's strain Y-30462, and the name *mulundensis* should be retained as the species epithet to maintain continuity with the previous body of work on the

Figure 4 Identification of mulundocandins in fermentations of *A. mulundensis* DSMZ 5745. (**a**) Identification of mulundocandin (**1**) and deoxymulundocandin (**2**) by high-resolution MS. (**b**) HPLC UV chromatograms demonstrating that mulundocandins are localized in the mycelium and not excreted into the culture broth. See Material and methods for extraction and fermentation conditions. A full color version of this figure is available at The *Journal of Antibiotics* journal online.

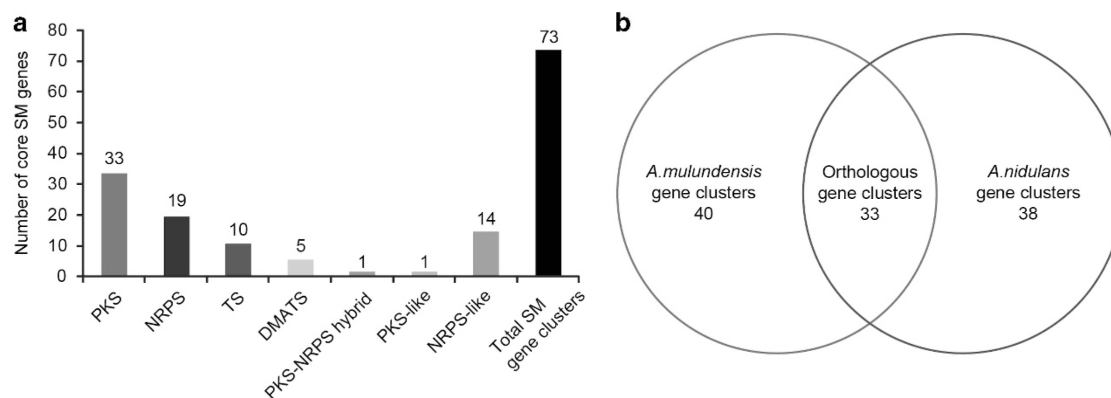

Figure 5 Analysis of secondary metabolome of *A. mulundensis*. (**a**) Numbers of core secondary metabolite gene clusters and core genes predicted by anti-SMASH. (**b**) Venn diagram comparison of secondary metabolomes of *A. mulundensis* and *A. nidulans* FGSC A4. PKS, polyketide synthase; NRPS, non-ribosomal peptide synthetase; TS, terpene synthase; DMATS, dimethylallyl tryptophan synthase.

mulundocandin-producing fungus. The new species shares small penicillate heads with *A. sydowii* and *A. asperescens*, whereas it shares the general aspect of the aspergilla of *A. nidulans*. *Aspergillus mulundensis* shares Hülle cells with most other species in section *Nidulantes*. The greenish conidial pigments apparently have the same biogenic origin as those in *A. nidulans* (Supplementary Figure S1). We do not know the history of culture transfers and storage conditions of the type strain prior to its accession to the DSMZ. However, the apparent loss of the ability to form Hülle cells and the tendency of the strain to sector and grow as poorly sporulating and unpigmented mycelium suggest that the original wild-type isolate may have suffered excessive transfers or perhaps even may have been mutated to select for sub-strains with increased titers of mulundocandins. The DNA sequences, extrolites and gene clusters identified in this study should aid in the future recognition of new strains of *A. mulundensis*.

The type strain of *A. mulundensis* produces a unique combination of extrolites. It shares the dibenzofurans with *A. multicolor*, *A. karnatakaensis*, *A. aeneus* and echinocandin-like metabolites with *A. pachycristatus*, *A. cleistominutus*, *A. microthecius*, *A. navahoensis*, *A. quadrilineatus* and *A. rugulosus*. However, among the echinocandins described to date from Aspergilli, mulundocandin is unique because of its combination of a 12-methymyristoyl side and serine in the fifth position of the core peptide. Comparison of its genome with that of *A. nidulans* FGSC A4 also predicts the type strain can produce emericellamides, asperfuranones alternariol or related isocoumarins, nidulanins and fungisporins, orsellinaldehyde, orsellinic acid and derivatives, asperthecin and the siderophore ferricrocin. *Aspergillus mulundensis* also shares the emericellamide, asperthecin and ferricrocin gene clusters with *A. ustus* of subgenus *Nidulantes*, Sect. *Usti*.[37] Also significantly, *A. mulundensis* lacks the genetic machinery to produce sterigmatocystin, penicillins and several other prominent extrolites characteristic of *A. nidulans*.

It is worth pointing out that the standardized method for extrolite production and extraction from agar plugs[17] did not detect mulundocandins, yet they were readily produced using an approximation of fermentation conditions published in a previous patent.[16] Our analysis of the separated culture broth and mycelium indicated that mulundocandins were only present in the mycelium and not in the liquid (Figure 4), a pattern also observed in the pneumocandins, thus, suggesting that echinocandin metabolites are stored intracellularly and not secreted. This pattern of mycelial associated metabolite sequestration is also characteristic many other fungal metabolites.[38–40] Thus, considering the potential biological importance of the echinocandins to the fungi that produce them and that several species in sect. *Nidulantes* are known to readily produce echinocandins, future comprehensive analysis of sect. *Nidulantes* should also include methods specifically to detect whether strains produce echinocandins or not, either by using previously described chemotyping and bioassay methods,[10,41] or by using a genetic method that would specifically amplify sequences unique to echinocandin NRPS genes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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