The Journal of Antibiotics (2017) 70, 304–307

www.nature.com/ja

NOTE

Re-identification of the ascofuranone-producing fungus *Ascochyta viciae* as *Acremonium sclerotigenum*

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The Journal of Antibiotics (2017) **70,** 304–307; doi:10.1038/ja.2016.132; published online 2 November 2016

Ascofuranone (AF)[1] is a meroterpenoid with strong inhibitory activity against cyanide-insensitive alternative oxidases,[2–4] and with therapeutic efficacy against rodent infection models of African trypanosomiasis[5] and cryptosporidiosis.[6] This promising drug candidate was initially found from nitrosoguanidine-induced mutant No. 34 of a soil fungus,[1,7] which had been identified as *Ascochyta viciae* Libert (Dothideomycetes; sometimes incorrectly spelled *Ascochyta visiae*). A structurally related compound, ascochlorin (AC), is produced by the same source,[1,8] although these compounds have significantly different effects on the mammalian respiratory chain.[9] Therefore, cost-effective selective production is necessary to enable the use of AF as treatment against African trypanosomiasis, a neglected tropical disease that affects economically disadvantaged patients, many of whom may be unable to afford expensive treatments.

The mechanism of metabolic partitioning into AF and AC is an obvious key to achieving selective production. These compounds are supposed to be synthesized through prenylation of orsellinic acid and terminal cyclization via epoxidation,[7,10] but details of their biosynthesis have not been established. In theory, comparative genomics and transcriptomics of a set of closely related fungal strains that produce AF and AC at different ratios should help elucidate the biosynthetic pathway and the underlying genes. Although AF and AC derivatives have been reported from various distantly related filamentous fungi,[7] they generally lack molecular information and thus no such set is available. In this light, we examined the original AF-producing strain and sought closely related strains as a first step toward elucidating the biosynthetic pathway.

The AF-producing strain F-1392, a descendant of the original AF-producing mutant No. 34, was kindly provided by aRigen Pharmaceuticals (Tokyo, Japan). First, F-1392 was inoculated on oatmeal agar plates to promote sporulation,[11] and examined under stereo- and light microscopes. Colonies were white and floccose in appearance, and reached diameters of 20 mm in 10 days at 20 °C in continuous darkness (Figure 1a). Hyphae were 1–2 μm in width and were frequently fasciculated (Figure 1b). Phialides were formed from substratum and fasciculated aerial hyphae, and were solitary, orthotropic, awl-shaped without branching, 30–44 μm in length (Figure 1c). Small collarettes can be observed at the tip of phialides (Figure 1d). Conidia were hyaline, cylindrical, 4–7 × 1.2–1.8 μm, were formed at the tip of phialides and were aggregated in slimy heads (Figure 1e). Pycnidia were not formed and conidia were aseptate, in sharp contrast to the original identification as *Ascochyta viciae*, in which larger septate conidia are formed in pycnidia.[12] In fact, strain F-1392 was consistent with the morphology of *Acremonium sclerotigenum*, which is described in Gams' monograph.[13] Sclerotia, the characteristic structure of *Ac. sclerotigenum*, were not formed probably because strain F-1392 has been mutagenized and cultured over decades.

To corroborate the identification by morphology, we amplified internal transcribed spacers (ITS) and fragments of the large and small subunit (LSU and SSU) ribosomal RNA from genomic DNA extracted from F-1392 mycelia. PCR products were directly sequenced and chromatograms were visually inspected to ensure absence of contamination and PCR artifacts. After trimming of primer-derived and ambiguous terminal nucleotides, 1005-, 535-, and 1,283-bp sequences were obtained for SSU (DDBJ/ENA/GenBank accession number LC063775), ITS (LC063776) and LSU (LC063777) amplicons, respectively. We then obtained ribosomal sequence information from additional strains, namely CBS 124.42 and 451.68 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands), which were obtained with permission under the Plant Protection Act of Japan. CBS 124.42 is the nomenclatural type strain of *Ac. sclerotigenum*, and we resequenced the ribosomal amplicons since the existing database entries are rather short. The sequences obtained for SSU (LC144891), ITS (LC144892) and LSU (LC144893) amplicons were identical to those of F-1392 (Supplementary Table 1). On the other hand, CBS 451.68 was sequenced since molecular information is unavailable for *As. viciae*, and it is the only available strain established from the same origin as the type specimen. The 1007-, 503-, and 1294-bp fragments for SSU (LC063772), ITS (LC063773) and LSU (LC063774) showed no or just single-base mismatches to corresponding entries for *Ascochyta fabae* (Supplementary Table 1). We note that *Acremonium* and *Ascochyta* sequences show pronounced divergence, in line

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Received 16 March 2016; revised 16 September 2016; accepted 30 September 2016; published online 2 November 2016

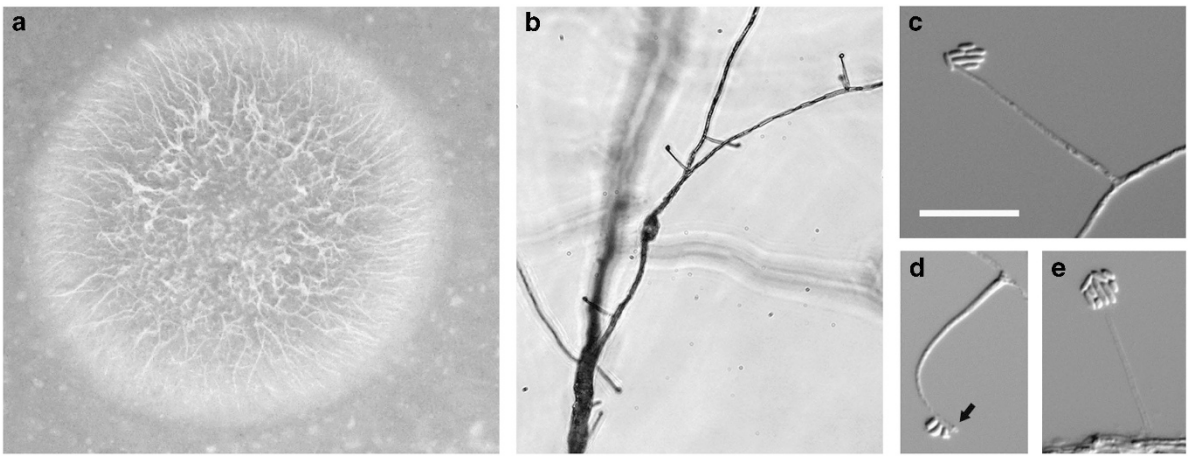

Figure 1 Morphology of strain F-1392. (**a**) Colony appearance. (**b**) Phialides forming on fasciculated aerial hyphae on a slide culture, as imaged under a stereomicroscope. (**c**–**e**) Phialides with conidia on a slide culture, as imaged under a light microscope with differential interference contrast. Bar, 20 μm; arrow, collarette.

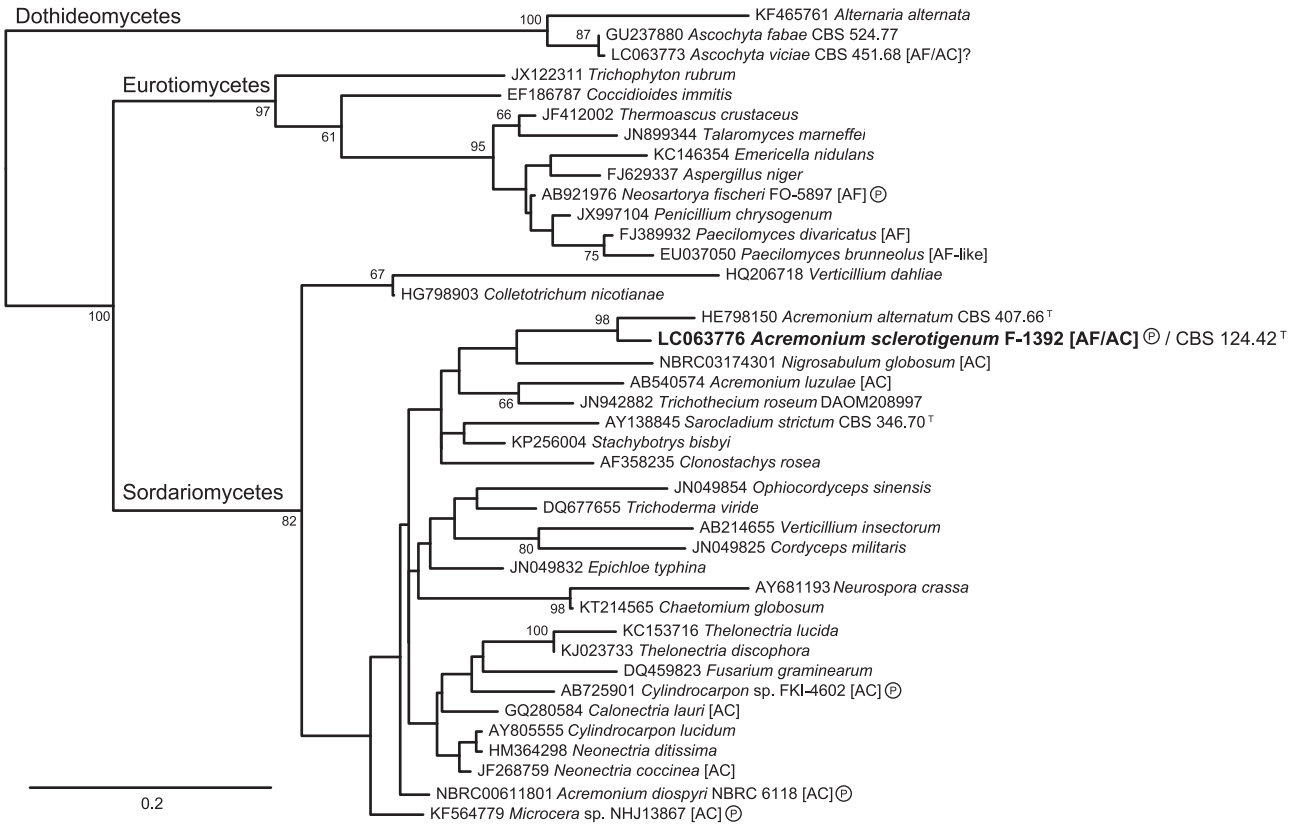

Figure 2 Phylogeny of fungi producing AF and AC. Unrooted tree constructed from ITS amplicon using PhyML version 20160605. The tree was constructed from a matrix of 38 OTUs and 309 sites using a GTR substitution model assuming four discrete gamma categories. DDBJ/ENA/GenBank accession numbers are indicated for each OTU, and those prefixed with NBRC were obtained from NBRC Culture Catalogue (http://www.nite.go.jp/en/nbrc/cultures/nbrc/online/). Species reported to produce AF or AC are indicated as such, and a superscript T and a circled P indicate nomenclatural type material and the producer strain, respectively. See text for AF production in *As. viciae*. Numbers adjacent to nodes indicate bootstrap support (100 replicates), although values below 50% are omitted. The scale bar indicates the number of substitutions per site.

with class-level taxonomic separation (Supplementary Table 1). Phylogenetic analysis of ITS amplicons was performed to show relationships among AF- and AC-producing fungi, including strain F-1392 as well as several species with perfect match entries reported by a BLAST search (data not shown). Among type or definitively identified strains, only *Ac. sclerotigenum* CBS 124.42 was identical to strain F-1392 (Figure 2), suggesting that other perfectly matched entries with different identification are for misidentified strains. Thus, the morphological (Figure 1) and molecular characteristics (Figure 2) of the AF-producing strain F-1392 match those of *Ac. sclerotigenum*.

Finally, AF and AC production was examined under the same fermentation conditions as originally published,[1,8] except that we grew

cultures in a 500-ml baffled conical flask containing 50 ml medium, with agitation at 200 rpm on a rotary shaker. AF and AC were extracted with methanol and analyzed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) fitted with a Synergi Hydro-RP ODS column (4 μm, 2 mm I.D. × 150 mm; Phenomenex, Torrance, CA, USA) based on absorbance at 295 nm. AF and AC were produced in strain F-1392 at 2.6 mg and 6.1 mg l^{-1} culture, respectively, but were not detected in methanol extracts of *As. viciae* CBS 451.68 (Supplementary Table 2). The limits of quantitation were 8 and 4 μg l^{-1} culture, respectively. Different strains of *Ac. sclerotigenum*, NBRC 5706 and 8385 (Biological Resource Center, National Institute of Technology and Evaluation, Tokyo, Japan), were examined to investigate their variation in AF and AC production. Ribosomal amplicons were sequenced (LC063778 through LC063783) and found to be identical to those of strain F-1392. These strains also produced both AF and AC, although production levels differed considerably (Supplementary Table 2). The AF/AC ratio was 0.43 for F-1392, 0.29 for NBRC 5706 and 0.69 for NBRC 8385 (Supplementary Table 2).

The genus *Acremonium* Link is an anamorph-typified genus currently encompassing more than 100 species[14] of distantly related filamentous fungi with simple morphology.[15] Fortunately, *Ac. sclerotigenum* CBS 124.42 is closely related to the ex-epitype strain of the type species *Ac. alternatum*, as shown in a recent large-scale phylogenetic study.[16] Thus, strain F-1392 undoubtedly belongs to genus *Acremonium*. On the other hand, species-level identification remains somewhat problematic because some species including *Ac. sclerotigenum* have been suggested to be conspecific.[11] Hence, rigorous investigation is required to establish conspecificity and to conclusively identify to species. However, such an investigation was far beyond the scope of this study, as we aimed only to find closely related strains that may be compared against each other in order to identify genes involved in AF biosynthesis. We therefore state only that strain F-1392 is *Acremonium sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams based on the observed morphology as well as ribosomal RNA sequence identity to the ex-type strain CBS 124.42.

AF and AC were first identified by the late Prof. Tamura and colleagues in a strain originally identified as *As. viciae*.[1,8] Unfortunately, the basis of identification was not published, and the patent gazette[17] only indicates the color of mycelia and the apparent lack of spores. It is therefore difficult to say why the descendant strain F-1392 is not *As. viciae*. *Ascochyta* and *Acremonium* are both mitosporic fungi, the morphological identification of which is generally difficult; however, these have so different morphology, in our opinion, that it is almost impossible to wrongly identify *Ac. sclerotigenum* as *Ascochyta*. Alternatively, it is possible that the fungal culture was contaminated with a small quantity of *Ac. sclerotigenum*, before or after the initial identification, since this species is widespread as a soil fungus and is also found in house dust.[18] Tamura and colleagues screened fungal strains to identify antiviral producers,[8] so contaminating *Ac. sclerotigenum* may have been unintentionally selected for because of the antibiotic activity of AC.

Does *As. viciae* have the ability to produce AF or AC? In our experiments, *As. viciae* CBS 451.68 did not produce either AF or AC under Tamura's conditions (Supplementary Table 2). In the literature, metabolites of *Ascochyta* species, such as the polyketide ascochitine[19] and the macrolide ascotoxin,[20] have been extensively studied due to phytopathogenicity. Nevertheless, AF and other AC-related compounds have never been reported from other *Ascochyta* species. These observations suggest that *As. viciae* does not produce AF and AC, and that we should consider all literature on AF and AC production in *As. viciae* as actually referring to AF and AC production in *Ac. sclerotigenum*, unless the fungi were explicitly identified in the course of the same analysis.

In phylogenetic analysis (Figure 2), species reported to produce AF- and AC-related compounds were scattered throughout three different classes. AF- and AC-producing strain F-1392 studied here belongs to Sordariomycetes. Interestingly, reports of AF and AC production appear to be biased towards Eurotiomycetes and Sordariomycetes, respectively. Several species within Eurotiomycetes are reported to produce AF and *Neosartorya fischeri* FO-5897, in particular, is definitively identified by molecular analysis.[21,22] In contrast, *As. viciae*, the only Dothideomycete reported to be a producer, is a dubious producer as noted. Hence, it is now evident that AF production is distributed in at least two distinct lineages: Sordariomycetes and Eurotiomycetes. This trait may have been horizontally transferred from one to the other or vertically inherited from a common ancestor. This point will probably be clarified by phylogenetic analysis of underlying genes yet to be identified.

The 2015 Nobel Prize in Physiology or Medicine awarded to Drs. Campbell, Ōmura and Tu highlights the importance of natural products as a source of drug against infectious diseases. In this manuscript, we demonstrated that the AF-producing strain F-1392 is *Ac. sclerotigenum* and we have successfully selected other AF-producing strains by virtue of this identification. These strains differ in AF production level and AF/AC ratio despite identical ribosomal DNA sequences, suggesting that these are suitable for comparative genomics and transcriptomics. Indeed, we have already performed such studies, and identification of genes that drive AF biosynthesis is now under way. We anticipate that the elucidation and manipulation of metabolic partitioning into AF and AC will lead to cost-effective, large-scale selective production of AF, as was achieved for avermectin.[23]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work is financially supported by JSPS KAKENHI Grant Numbers 21790402 (to YK), 23117004 (to MM) and 26253025 (to KK), and by JST/JICA Science and Technology Research Partnership for Sustainable Development (SATREPS; no. 10000284 to KK). We also acknowledge support from Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN), and from Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry. The authors thank Dr Masaichi Yamamoto (aRigen Pharmaceuticals) for providing fungal strain F-1392 and Dr Takashi Nakada (Keio University) for valuable comments on the manuscript.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)