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The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm?

EILIDH MOWAT*, CRAIG WILLIAMS†, BRIAN JONES‡, SUSAN MCCHLERY§ & GORDON RAMAGE§

**Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, †Microbiology Department, Yorkhill Hospital, ‡Glasgow Royal Infirmary, and, and §Section of Infection and Immunity, Glasgow Dental School, Faculty of Medicine, University of Glasgow, Glasgow, UK*

Aspergillus fumigatus is an increasingly prevalent opportunistic fungal pathogen of various immuno-compromised individuals. It has the ability to filament within the lungs forming dense intertwined mycelial balls. These morphological characteristics resemble those of microbial biofilms, which are matrix enclosed microbial populations, adherent to each other and/or to surfaces or interfaces. The purpose of this paper is to review some recent experiments that indicate the potential biofilm forming capacity of *A. fumigatus in vitro*. Initially it was established that conidial seeding density is important for stable biofilm development. In the optimized model conidial germination and filamentous growth characteristics were not observed until 8 h, after which a multi-cellular population expanded exponentially forming a thick structure (approx. 250 μm). Calcofluor white staining of this revealed the presence of extracellular polymeric matrix material, which increased as the biofilm matured. Subsequent antifungal sensitivity testing of this structure showed that azoles, polyenes and echinocandins were ineffective in reducing the cellular viability at therapeutically attainable concentrations. Microarray and real-time PCR analysis demonstrated the up-regulation of AfuMDR4 during multicellular growth and development, which may account the recalcitrance observed. Overall, *A. fumigatus* appears to possess the classical elements of biofilm growth, namely multicellularity, matrix production and sessile resistance. This controversial approach to understanding the biology of *A. fumigatus* infection may provide crucial information on how to treat this pathogenic fungus more effectively.

Keywords *Aspergillus fumigatus*, biofilm

Introduction

Aspergillus species are opportunistic filament forming moulds comprising over 180 different species, of which *Aspergillus fumigatus* causes the majority of human aspergillus infections [1,2]. *A. fumigatus* is now the second most common fungal infection found in hospitalized patients, after *Candida albicans* [3]. *A. fumigatus* is ubiquitous, with a worldwide distribution due to the production of small spores called conidia that have an average size of 2–3.5 μm, resulting in the conidia dispersing in the air and remaining in the atmosphere for prolonged periods [4]. Following inhalation these conidia are usually efficiently eliminated by host immune factors. However, in immuno-compromised patients *A. fumigatus* can cause a range of systemic diseases with mortality rates ranging from 30–90% [5–7]. Pulmonary infection may also occur in other patients such as those with cystic fibrosis (CF). In these

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Correspondence: Gordon Ramage, Section of Infection and Immunity, Glasgow Dental School, Faculty of Medicine, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK. Tel: +44 (0) 141 211 9752; Fax: +44 (0)141 353 1593; E-mail: g.ramage@dental.gla.ac.uk

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patients, infection with *A. fumigatus* may cause allergic bronchopulmonary aspergillosis (ABPA), invasive disease (IA) or a fungus ball (aspergilloma) type aspergillosis [8,9].

Filamentous growth is an important morphological characteristic of *A. fumigatus* and is particularly pivotal during the development of an aspergilloma (mycetoma). The initial establishment of these chronic *A. fumigatus* infections involves the germination of conidia and subsequent hyphal invasion of the lung tissues [10]. Histology and microscopic examination of bronchopulmonary lavage (BAL) samples has revealed the presence of numerous *A. fumigatus* hyphae in the form of a complex multicellular mycetoma structure, which are in essence similar to the morphologically diverse biofilms formed by *Candida* species [11,12]. In contrast to these complex intertwined structures formed by *Candida* spp., very limited information is currently available on the development and behaviour of *A. fumigatus* adherent multicellular communities, and their response to antifungal treatment. Although still an area of contentious debate, there are limited reports suggesting that *Aspergillus* species are able to grow and develop structures which by definition are microbial biofilms [13,14].

The purpose of this paper is to review the current understanding of pathogenic *Aspergillus* species growing as multi-cellular structures. Our recent publication in the *Journal of Medical Microbiology* will be used as the basis for this discussion [15]. This will also include information relating to some new and ongoing studies of *Aspergillus* biofilms, investigating their characteristics from both a phenotypic and genotypic perspective, and relating this to antifungal treatment failure

What are biofilms?

Our generalized perception of microorganisms as free-floating unicellular life forms is primarily based on the pure-culture mode of growth. This mode of growth has overwhelmingly predominated in the study of microbial physiology and pathogenesis in the research laboratory since microorganisms can be diluted to a single cell and studied in liquid culture. In spite of this, the majority of microbes in their natural habitats persist attached to surfaces within a structured biofilm ecosystem and not as free-floating (planktonic) organisms [16]. Biofilms can be defined as structured microbial communities attached to surfaces and encased within a matrix of exopolymeric materials [17]. This life-style results in phenotypic characteristics that are markedly different from that of their planktonic existence, such as increased resistance to anti-microbial agents and protection from host defences. Conservative estimates suggest that 65% of all human microbial infections involve biofilm formation [16]. A recent surge of research activity has taken place with an increasing recognition of the role that microbial biofilms play in human medicine. In fact, over the past 10 years there has been a 2000% increase in peer reviewed publications relating to biofilm research activities based on citations in PubMed (www.pubmed.gov). Bacterial biofilms are the mainstay of this research activity, with *Candida* biofilms comprising of only 4.11% of this in 2006. When a search of '*Aspergillus* biofilms' is undertaken using this search engine, less than 20 publications are cited, which include the following [13–15,18–20]. Whether this is due to inactivity within the field or an issue of nomenclature this remains to be seen. For example, work by the Gutiérrez-Correa group described *A. niger* biofilms in terms of surface adhesion fermentation, i.e., solid state fermentation, rather than as clinical biofilms *per se* [18]. Nevertheless, recent studies by our group have given us reason to believe that *Aspergillus* multicellular masses are medically important and exhibit attributes that may make treatment of these infections problematic, similar to that of biofilms [21].

Does *Aspergillus* have the capacity to form a biofilm?

Whether *A. fumigatus* can form a biofilm was the focus of a recent mini review, which discussed its potential ability to satisfy key biofilm characteristics [22]. The classical definition of a biofilm is a *matrix enclosed microbial population adherent to each other and or to surfaces and interfaces.* In our preliminary studies we explored whether an *A. fumigatus* multicellular structure (or mycelial mass) fitted this definition. Our previous studies with *C. albicans* demonstrated that filamentation was a requisite for successful biofilm formation. It was shown that single and double knockout mutations in the *efg1* and *cph1* signal transduction genes, which are involved in morphological differentiation, altered the dimorphic behaviour and subsequent ability to adhere and form coherent biofilm structures [23,24]. Although the life cycles of these two pathogenic fungi are significantly divergent, cellular differentiation and filamentation are a key aspect to their pathogenic potential, therefore it seemed logical to investigate *A. fumigatus* as a sessile organism.

A key factor we observed early in our studies was the critical importance of conidial seeding density, a phenomenon described previously by Villena and Gutierrez-Correa (2006) for *A. niger* biofilms [14].

Clearly, structural morphology and integrity of these multi-cellular structures was dependant upon the concentration of conidia per millilitre of medium, a phenomenon previously identified with *C. albicans* biofilm development [11]. It was demonstrated that both the metabolic activity and the biomass of the biofilms exhibited a positive correlation to the conidial seeding density. However, based on confocal microscopy the optimal conidial concentration was determined as 1×10^5 per millilitre. Fig. 1 illustrates the morphological complexity of the mature structure. This structure was inherently more stable, reproducible and amenable to high throughput testing that is required for the screening of clinical isolates and defined mutants, or for testing the susceptibility of antifungal agents [15]. This concentration differs by from work by Villena and Gutiérrez-Correa (2006), who reported an optimized condial density of 1×10^6 per millilitre [14]. However, this ten fold increase is due to the differences within the biofilm model systems, which utilise different substrates and media.

How do these structures develop?

Fungal biofilms, like bacterial biofilms, have defined developmental phases [16,25]. These key phases include arrival at an appropriate substrate, adhesion, colonization, polysaccharide production and biofilm maturation, and dispersal [26]. When we consider *C. albicans* catheter-related biofilm infections, a yeast cell is first inoculated onto the luminal surface of their catheter, either from endogenous or exogenous flora, which adheres to the substrate quickly forming hyphae. A filamentous intertwined framework develops, consisting of yeast cells, pseudohyphal forms and true hyphae, which gradually become encased within a polymeric matrix [12,25,26]. Subsequently, yeast cells can bud and detach from the matrix, either colonizing an adjacent region of the catheter or disseminating into the bloodstream [12,25]. We therefore studied the developmental processes related to *A. fumigatus* multicellularity with an optimized conidial concentration.

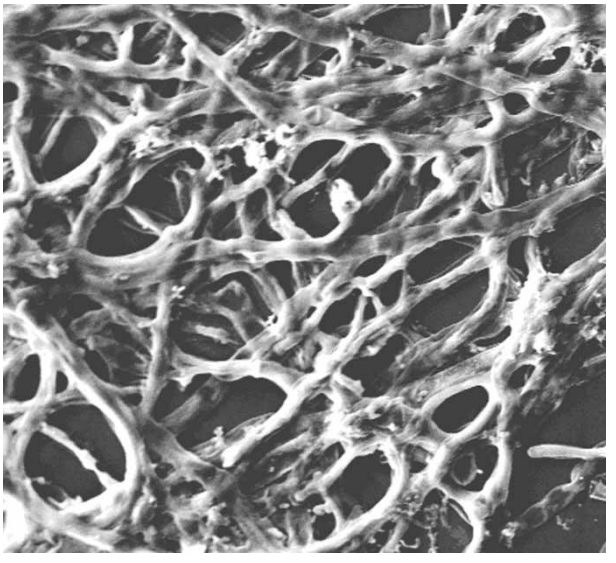

Fig. 1 *Aspergillus fumigatus* Af293 biofilm grown in RPMI medium over 24 h at 37°C on a Thermanox™ coverslip. Note the intertwined filamentous networks that maintain biofilm stability and integrity.

The kinetics of *C. albicans* intertwined filamentous multicellular development are more rapid than for *A. fumigatus*, although the generic characteristics remain similar [25]. Following initial conidial seeding there is a lag phase, where conidial adhesion occurs. The conidia begin swell and germinate, forming mycelia after 6 to 8 h. Hyphae then intertwine forming a monolayer (8 to 12 h), followed by an increased structural complexity and three-dimensional architecture over a subsequent 4 to 8 h, which then reach a plateau as biofilm development ceases and a steady state occurs (24 h). This is illustrated in biofilm depth measurements assessed by confocal microscopy over selected time intervals, which reveal the kinetics of biofilm growth (Fig. 2). This pattern closely resembles the developmental kinetics of both *C. albicans* and *A. niger* biofilm growth, in terms of the phase and associated morphologies [11,19]. This may provide an advantage to *A. fumigatus* during the progression of disease in susceptible patient groups, providing the ability of the conidia to germinate to form mycelial masses (mycetoma), which then latterly have the ability to penetrate the pulmonary epithelium prior to angioinvasion and systemic spread [10,27].

When these observations are now considered in terms of the definition of a biofilm, we have demonstrated *A. fumigatus* as an adherent, intertwined and multi-cellular fungi. However, our previous study did not demonstrate the presence of matrix material, an integral feature of microbial biofilms [28]. This was a key observation in a recent critical review entitled 'Do *Aspergillus* species produce biofilm?' [22]. Recent work to answer this critique was performed using Calcofluor white staining technique, a fluorescent dye that stains extrapolymeric matrix material. Kuhn and coworkers (2002) previously used this technique successfully to demonstrate the presence of matrix with various species of *Candida* [29]. In our investigations we have shown the presence of matrix material as diffusely distributed amongst the filamentous growth (Fig. 3). In addition, this extent of staining increases in a time dependent manner. Further experiments to quantify and qualify

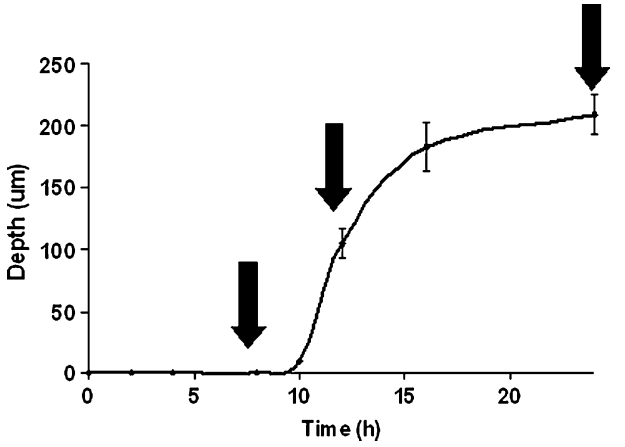

Fig. 2 *Aspergillus fumigatus* Af293 was seeded at 1×10^5 conidia/ml of RPMI onto Thermanox™ coverslips. Multi-cellular biofilm growth was examined using a confocal scanning laser microscope to assess the depth of the biomass using a *z*-stack function after 2, 4, 6, 8, 10, 12, 16 and 24 h. Note the lag phase for 10 h, followed by an exponential increase in depth (10–18 h), followed by a plateaux (24 h). Arrows indicate sampling points of RNA harvesting for microarray analysis.

the nature of the matrix are currently under investigation. Nevertheless, several recent published studies have also shown the presence of extrapolymeric material using their specific models. Beauvais and colleagues (2007) elegantly demonstrated the presence of extracellular hydrophobic matrix composed of galactomannan, alpha-1,3 glucans, monosaccharides and protein moieties in an aerial static *A. fumigatus* biofilm model [13]. This model was developed to study the characteristics of a fungus ball, opposed to using the typical submerged shaking culture system. Within the ball, hyphae are agglutinated and collectively form a macrocolony of highly branched hyphal elements that are tightly associated with one another. This study demonstrated that hydrophobic matrix cohesively bound hyphae together, and that the matrix increased with maturity of the developing structure. In addition, Villena and Gutierrez-Correa (2007) recently described an *A. niger* submerged biofilm system in which extrapolymeric material was produced [19]. In this system an extracellular matrix surrounding germ tubes and hyphae was observed. Biofilm mycelia were orderly distributed forming surface and inner channels. This was in stark contrast to pellets, which were composed of highly intertwined superficial hyphae and a densely packed deep mycelium. Collectively these studies report the presence of a polymeric material associated with multicellular filamentous growth, but as its role has to be clearly defined. By definition, however, *A. fumigatus* does appear to have the ability to form structures of a biofilm characteristic, and importantly exhibit properties consistent with all other microbial biofilm systems, i.e., resistance to antimicrobial challenge [13,21].

Antifungal resistance

It is well know that fungal biofilms such as *Candida albicans* exhibit increased resistance to antifungal agents [30,31]. The complex structure, matrix material and efflux pumps all play defined roles in resistance to azoles and polyenes [25,28,32,33]. Beauvais and coworkers (2007) demonstrated resistance to polyene antifungal agents in their *A. fumigatus* biofilm model, but sensitivity to triazoles and echinocandin exposure [13]. In our recent paper, we used the CLSI M38-A methodology to compare susceptibility profiles of azoles, polyenes and echinocandins to sessile populations using an XTT colometric based assay system.

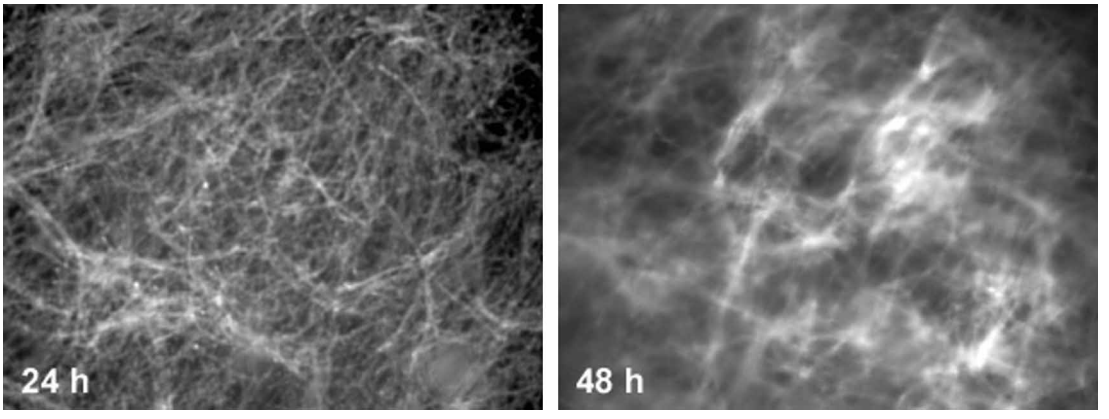

Fig. 3 Extrapolymeric matrix production by *Aspergillus fumigatus* biofilms. *Aspergillus fumigatus* Af293 was seeded at 1×10^5 conidia/ml of RPMI onto Thermanox™ coverslips and grown for 24 and 48 h. Biofilms were stained with Calcofluor white (Molecular probes) and viewed using a confocal scanning laser microscope at 10 ×. Note the diffuse staining of the 24 h biofilms, which increases with intensity with the 48 h biofilms. Polymeric material is also noted attached to hyphae, as previously described [13].

This data showed that these agents were up to 1000 times less effective against multicellular filamentous structures than against germinating conidia [15]. Although the densities of cells used in these assays are significantly different, clinically aspergillus infections are unlikely to be characterised by small quantities of germinating conidia used in the M38-A assay. Therefore, this methodological approach is justified and demonstrates how antifungal effectiveness may be modified by the extent of filamentous growth and increase in total biomass, as described previously [34,35]. It has also been previously reported that varying inoculum sizes can adversely affect antifungal susceptibility testing results [36,37]. Some recent preliminary work by our group has shown that if the biofilm structure is treated earlier during its development then antifungal challenge is more effective. For example, voriconazole MIC's were shown to increase from 0.5 mg/l to >256 mg/l when challenged at 8 and 24 h, respectively [21]. Early intervention with antifungal therapy for *A. fumigatus* infection may be crucial to improving the likelihood of a successful outcome with this agent; a finding supported by a number of clinical studies [38,39]. This may be related to the fact that antifungals delivered earlier prevent the organism from developing into a mycelial mass, a structure which is generally unresponsive to antifungal therapy. Like *C. albicans*, the mechanism of antifungal resistance within these structures is a multi-factorial process, and will be underpinned by complex molecular events.

Potential molecular mechanisms of resistance

Although transcriptomics is in its relative infancy within the field of aspergillosis, molecular tools have become available to begin unravelling the genetic factors involved in this complex process. Microarrays containing the entire genome of various strains of *A. fumigatus* are now available for transcriptome analysis under various conditions. A recent study investigated the response of *A. fumigatus* to short exposures (30, 60, 120 and 240 min) of voriconazole [40]. In this study it was revealed that ergosterol biosynthesis gene transcripts were down-regulated and that ABC multidrug transporters and MFS transporters were up-regulated. The up-regulation of efflux pumps in *C. albicans* is a well defined azole resistance mechanism and is also associated with the biofilm growth in a phase dependant manner [33,41,42]. Recent microarray analysis of three phases of *A. fumigatus* (Af293) multicellular growth (8, 12 and 24 h), as illustrated in Fig. 2, was performed by our group using biochips developed in Toulouse (http://biopuce.insa-toulouse.fr/). This data indicated that transcripts of AfuMDR4, a member of the ATP-binding cassette superfamily, were up-regulated during exponential multicellular growth (12 h), and this remained up-regulated in the latter phases of growth (24 h) in comparison to earlier 8 h growth. Quantitative real-time PCR analysis of three clinical *A. fumigatus* isolates showed a similar pattern of expression (Fig. 4). Therefore, this gene may explain the high level azole resistance we detect *in vitro*. Interestingly, previous studies have shown an association between AfuMDR4 and itraconazole resistance [43]. The role of these efflux pumps in *A. fumigatus* multicellular biofilm formation has yet to be deduced, but based on data from other micro-organisms efflux pumps are likely to be up-regulated as a means of cellular detoxification. The high cell density within this environment will result in a build up of toxic secondary

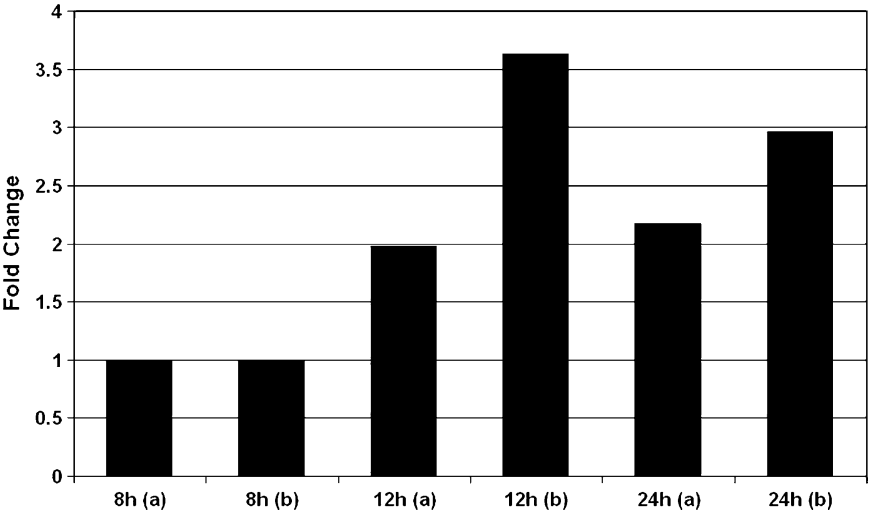

Fig. 4 AfuMDR4 differential expression during *Aspergillus fumigatus* biofilm development. *Aspergillus fumigatus* Af293 and three clinical isolates were seeded at 1×10^5 conidia/ml of RPMI onto 75 cm^2 tissue culture flasks and grown for 8, 12 and 24 h. Total RNA was extracted and cDNA produced for differential expression analysis using microarrays and real-time PCR. Note that as the biofilm matures the expression of AfuMDR4 increases greater than 2-fold after 12 and 24 h. This was observed for both microarray analysis (a) of Af293 and real-time PCR (b) of Af293 and three clinical strains.

metabolites, therefore the high expression of these pumps is likely, and may serendipitously confer antifungal resistance.

What is the clinical significance of *Aspergillus* biofilms?

A. fumigatus is now a leading fungal pathogen, and one of the most significant opportunistic fungi in bone marrow transplant patients [44]. *A. fumigatus* is also found in a range of other patient groups, including cystic fibrosis patients, HIV-positive patients and other immuno-compromised individuals [45,46]. Its presence in CF patients has been described as a significant risk factor for another pulmonary biofilm organism, i.e., chronic *P. aeruginosa* infection [47]. The presence of mycetomas in the upper airways, the pulmonary epithelial cells of the alveoli, or in the maxillary sinuses provides compelling evidence that *A. fumigatus* biofilms are more prolific and problematic than once thought [1,10,48]. These infections are typified by intricate networks of hyphae that develop from inhaled conidia [9]. Morphologically these structures resemble other fungal biofilms, such as those of *C. albicans* [11] which are clinically important due to their role in pathogenesis and resistance to antifungal agents [15,30].

What does the future hold for *Aspergillus* biofilm research?

The data presented above illustrates that *A. fumigatus* has the ability to form multi-cellular communities that satisfy the broad terminology for a biofilm, illustrating the close relationship that exists between all microbial biofilm systems. Although the model system described herein relies on a plastic substrate on which to *A. fumigatus* adheres and germinates, the developmental processes of filamentation, dichotomous branching and multicellular growth do not appear to be influenced by the surface. Within the lung environment the substrates are significantly different. Nonetheless, preliminary data suggests that certain cell wall proteins are expressed in our biofilm model as they are in the lung tissue of an invasive aspergillosis guinea pig model (unpublished results). Whether those working within the aspergillosis community will embrace the term 'biofilm', or not, does not diminish the significance of the observations reported herein. The complex events involved in the developmental process of the multi-cellular community may provide pertinent information of how pulmonary infections can develop into, and proliferate from, a mycetoma towards an invasive aspergillosis, or alternatively inform us of potential drug targets for improved antifungal therapy. In addition, these studies may allow us to understand complex multi-cellular behaviour of simple eukaryotes, by understanding how cell-cell communication and population dynamics permits biofilm development.

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