Analysis of the *Aspergillus fumigatus* Biofilm Extracellular Matrix by Solid-State Nuclear Magnetic Resonance Spectroscopy

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*Aspergillus fumigatus* is commonly responsible for lethal fungal infections among immunosuppressed individuals. *A. fumigatus* forms biofilm communities that are of increasing biomedical interest due to the association of biofilms with chronic infections and their increased resistance to antifungal agents and host immune factors. Understanding the composition of microbial biofilms and the extracellular matrix is important to understanding function and, ultimately, to developing strategies to inhibit biofilm formation. We implemented a solid-state nuclear magnetic resonance (NMR) approach to define compositional parameters of the *A. fumigatus* extracellular matrix (ECM) when biofilms are formed in RPMI 1640 nutrient medium. Whole biofilm and isolated matrix networks were also characterized by electron microscopy, and matrix proteins were identified through protein gel analysis. The $^{13}$C NMR results defined and quantified the carbon contributions in the insoluble ECM, including carboxyls, aromatic carbons, polysaccharide carbons (anomeric and nonanomers), aliphatics, etc. Additional $^{15}$N and $^{31}$P NMR spectra permitted more specific annotation of the carbon pools according to C-N and C-P couplings. Together these data show that the *A. fumigatus* ECM produced under these growth conditions contains approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components, and up to 14% lipid. These fundamental chemical parameters are needed to consider the relationships between composition and function in the *A. fumigatus* ECM and will enable future comparisons with other organisms and with *A. fumigatus* grown under alternate conditions.

**A. fumigatus** is the most important etiological agent of human aspergillosis and is recognized as a highly allergenic and opportunistic pathogen, causing acute and chronic infections particularly among immunocompromised individuals (1). Hospital-associated outbreaks of *A. fumigatus* may occur during periods of construction or renovation (2) or when fungi colonize water distribution systems that then lead to spore aerosolization in patient care areas and patient exposure (1). Additionally, *A. fumigatus* can cause infections as a result of colonizing medical implant devices, including cardiac pacemakers, joint replacements, and breast implants (3). Despite improvements in diagnostics and the antifungal armamentarium, including broad-spectrum azoles and the echinocandin antifungals, mortality due to *A. fumigatus* invasive infections remains high.

Like many microorganisms, *A. fumigatus* can also assemble into multicellular communities, termed biofilms, composed of cells plus an extracellular matrix (ECM) (4–9). Although more work is needed to fully understand the functional implications of biofilm formation by *A. fumigatus*, recent evidence suggests that the ECM may provide the infrastructure for enhancing cell density, controlling disaggregation, and altering nutritional needs, as well as providing a protective physical and chemical barrier that can decrease sensitivity to competitors and to antifungal drugs and the immune response (10). Understanding the composition of microbial biofilms and the ECM is important for considering functional differences among biofilm formers and, ultimately, for developing strategies to inhibit biofilm formation. Valuable detail has been obtained for many biofilms regarding macroscopic properties and the identification of key matrix parts (5, 7, but quantitative compositional detail remains sparse for most biofilm systems (11).

Mature *A. fumigatus* biofilms have been observed as three-dimensional assemblies between 10 μm and 200 μm thick. The putative parts list for the *A. fumigatus* ECM includes polysaccharides (galactomannan, galactosaminogalactan, α-1,3 glucans, and monosaccharides), proteins (major antigens and hydrophobins), melanin, and extracellular DNA (8). These identifications relied primarily upon immunoeassays, yet suggested compositions vary depending upon the growth conditions and analysis methods. Attempts to generate quantitative descriptions of the *A. fumigatus* ECM have been limited to the soluble or low-molecular-weight components of the ECM. Indeed, the insoluble and complex nature of the ECM of most biofilms has posed a challenge to analysis by traditional techniques, which generally rely on dissolution of matrix parts and solution-based methods such as high-pressure liquid chromatography (HPLC)-mass spectrometry, protein analyses, and solution nuclear magnetic resonance (NMR) to examine soluble parts. Estimates of relative quantities of these components in the intact matrix are sometimes made. However, the
inability to completely solubilize ECM components and the possible perturbations or degradation of material that can occur during sample preparation can severely compromise estimates of composition for the intact ECM. Thus, despite the prevalent roles that biofilms play in human infection, there has been little reliable quantitative information available regarding biofilm matrix composition.

Solid-state NMR spectroscopy is uniquely suited to study complex and insoluble biological materials (12) such as whole cells (13–18), biofilms, and the extracellular matrix (19–22). Solid-state NMR approaches can be used to quantify composition and to measure internuclear distances to determine parameters of architecture in such macromolecular assemblies and does not require soluble or crystalline samples (15). We recently introduced two approaches to define the composition of the insoluble ECM of bacterial biofilms using solid-state NMR integrated with complementary biochemical analysis and electron microscopy. Both approaches involve a panel of one-dimensional NMR experiments, which can be employed on any spectrometer equipped to perform solid-state NMR measurements. In the case of a uropathogenic E. coli biofilm that assembles an amyloid-associated ECM, we employed a bottom-up approach and discovered that the spectral addition of only two separate samples corresponding to separate isolated biofilm parts, (i) curli amyloid fibers and (ii) a modified form of cellulose, in a 6:1 ratio, was able to completely recapitulate the spectrum of the intact ECM (20). This was the first quantitative determination of the composition of an intact ECM preparation. In this approach, one requires separate samples of putative biofilm parts for analysis, and these are not readily available for many biofilm systems. Thus, while working with the more compositionally complex biofilms formed by V. cholerae, we developed a top-down methodology that could be applied to other biofilm systems with only the complex ECM material to examine (21). In the top-down approach, carbon contributions are assigned by type of chemical functionality (e.g., carboxyls, aromatics, or anomerics) and quantified by peak integrals. If 15N labeling of the growth medium is possible, then nitrogen pools can also be examined. In addition, the carbons can be more specifically annotated through 13C-15N recoupling experiments to determine, for example, what percentages of carboxyls may be part of peptide bonds versus non-nitrogen-bonded carbonyls. Together, the analysis of the V. cholerae biofilm formed on a minimal agar medium revealed a complex ECM dominated by highly modified polysaccharides, with additional contributions from lipids and proteins.

Here we provide our analysis of the biofilm composition of the filamentous fungus A. fumigatus grown in RPMI 1640 medium, a medium commonly employed for mammalian cell culture, using the top-down methodology that requires only the complex matrix material isolated from the hyphae. We integrated electron microscopy, protein gel and protein identification, and quantitative solid-state NMR measurements involving 13C, 15N, and 31P nuclei to characterize the A. fumigatus biofilm. In order to permit 15N NMR measurements, we generated a fully 15N-labeled version of RPMI 1640 medium. Collectively, we provide a general spectral accounting of the carbon, nitrogen, and phosphorous contents and a detailed analysis of the carbon pools in the A. fumigatus ECM.

MATERIALS AND METHODS

Aspergillus growth and ECM extraction. A standardized Aspergillus fumigatus suspension was inoculated in each of five 500-ml polystyrene tissue-culture flasks containing 100 ml of RPMI 1640 medium (final concentration of 105 conidia/ml) and incubated at 37°C for 96 h. To generate a fully 15N-labeled version of RPMI 1640, all amino acids were replaced by 0.674 g/liter 15N-labeled algal amino acid mixture (Aldrich, catalog number 608947). The algal extract contains between 65 and 95% amino acids by mass and has an isoiline enrichment of 99% for 15N. For uniformly 15N-labeled samples, Ca(NO3)2 (98% 15N enrichment) was also used in place of its unlabeled counterpart. Harvested biofilms were washed in situ twice with 100 ml of phosphate-buffered saline (PBS) to remove planktonic and loosely adherent cells. The sessile cells were then flooded with 40 ml of 10 mM Tris (pH 7.4) per flask and mixed mildly with a vortex mixer for 60 s. Each sample was transferred to a 50-ml conical tube and stored at 4°C until processing.

For the ECM extraction, the tubes containing the A. fumigatus biofilm were subjected to sonication (Fisher Scientific, model FB505; amplitude of 20%) for 10 min, followed by high-speed mixing on a vortex mixer for 30 s. The tubes were then centrifuged at 3,600 × g at 4°C for 15 min. The supernatant was poured into new tubes and centrifuged again to remove additional cells. The resulting supernatant was dialyzed against distilled water for 2 days (3.5-kDa molecular-mass cutoff). The supernatant was flash frozen in liquid nitrogen and lyophilized.

EM. Negative-staining transmission electron microscopy (TEM) was performed on ECM samples. Samples were applied to 300-mesh copper grids coated with Formvar film (Electron Microscopy Sciences, Hatfield, PA) for 2 min and then rinsed in deionized water. The samples were negatively stained with 2% aqueous uranyl acetate for 90 s, excess stain was wicked off with filter paper, and then the sample was air dried. Microscopy was performed with a JEM-1400 instrument (JEOL, LLC) operated at 120 kV. For scanning electron microscopy (SEM) analysis, Aspergillus biofilms were suspended in 10 mM Tris (pH 7.4), fixed for 45 min with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed in the same buffer, and postfixed for 45 min with 1% aqueous OsO4. After dehydration in an ascending ethanol series (50, 70, 90, and 100% [twice]; 5 min each), samples were gently vacuum filtered onto 0.2- and 0.45-μm polyamide filters (Sartorius, Bohemia, NY) kept under a steady stream of 100% ethanol. Samples were then critical-point dried with liquid CO2 in a Tousimis Autosamdri-815B apparatus (Tousimis, Rockville, MD), mounted onto double-sided copper tape on 15-mm aluminum stubs (Electron Microscopy Sciences, Hatfield, PA), and sputter coated with 50 Å of gold-palladium using a Denton Desk II sputter coater (Denton Vacuum, Moorestown, NJ). Visualization was performed both with a Hitachi S-3400N SEM (Hitachi HTA, Dallas, TX) operated at 10 to 15 kV using Everhart Thornley secondary electron (SE) detection and with a Zeiss Sigma field emission SEM (FESEM). TEM and SEM detection at a working distance of 4 to 5 mm. Images were captured in TIFF using store resolution of 2,048 by 1,536 and a line averaging noise reduction algorithm.

SDS-PAGE protein analysis and identification. Lyophilized ECM was suspended in SDS-PAGE sample buffer containing 8 M urea and 50 mM dithiothreitol (DTT) and used for electrophoresis in a 4 to 20% gradient Tris-glycine SDS-polyacrylamide gel (Novex). For Edman degradation (UC Davis Proteomic Core), samples were transferred to a polystyrene membrane and were loaded onto an ABI Procise sequencer and subjected to Edman degradation. In this processing, for each residue/cycle, the free N terminus of each immobilized protein was coupled with phenyl isothiocyanate, cleaved with trifluoroacetic acid, and then converted to the more stable phenylthiohydantoin amino acid derivative, which was separated by reverse-phase chromatography and detected by UV-visible spectrophotometry.

Solid-state NMR. All solid-state NMR experiments were performed using an 89-mm wide-bore Varian magnet at 11.7 T (499.12 MHz for 1H, 13C, 15N, and 31P). The samples were spun at 4°C and in a 50 mM Tris (pH 7.4) buffer containing 15N-amino acid mixture at 37°C. The samples were placed in a Bomem broad-band triple-resonance CP/MAS probe and scanned at 200 MHz for 1H, 77.1 MHz for 13C, 40.0 MHz for 15N, and 51.3 MHz for 31P. NMR experiments were performed with a Bruker AVANCE 600 spectrometer equipped with a cryogenically cooled 7 mm triple-resonance CP/MAS probe. A series of pulse sequences (50, 70, 90, and 100% [twice]; 5 min each) were used with 1% aqueous OsO4. After dehydration in an ascending ethanol series (50, 70, 90, and 100% [twice]; 5 min each), samples were gently vacuum filtered onto 0.2- and 0.45-μm polyamide filters (Sartorius, Bohemia, NY) kept under a steady stream of 100% ethanol. Samples were then critical-point dried with liquid CO2 in a Tousimis Autosamdri-815B apparatus (Tousimis, Rockville, MD), mounted onto double-sided copper tape on 15-mm aluminum stubs (Electron Microscopy Sciences, Hatfield, PA), and sputter coated with 50 Å of gold-palladium using a Denton Desk II sputter coater (Denton Vacuum, Moorestown, NJ). Visualization was performed both with a Hitachi S-3400N SEM (Hitachi HTA, Dallas, TX) operated at 10 to 15 kV using Everhart Thornley secondary electron (SE) detection and with a Zeiss Sigma field emission SEM (FESEM). TEM and SEM detection at a working distance of 4 to 5 mm. Images were captured in TIFF using store resolution of 2,048 by 1,536 and a line averaging noise reduction algorithm.
RESULTS

A. fumigatus biofilm formation and ECM isolation and protein analysis. A biofilm contains both microbial cells and their secreted extracellular matrix (ECM) (23). The A. fumigatus biofilm was visualized by scanning electron microscopy (SEM) (Fig. 1A to C). The biofilm hyphae are densely packed and connected to one another by tightly woven webs of ECM. The ECM also appears as fibrous and granular networks coating the individual hyphae (Fig. 1C).

The biofilm hyphae are N-linked glycosylated, and digestion with peptide-N-glycosidase F (PNGase) enhanced the intensity of band 3 while depleting band 2 intensity (see Fig. S1 in the supplemental material). By Edman degradation, the protein in band 3 also contained the same N-terminal amino acids as Asp f2. Thus, band 3 represents deglycosylated Asp f2. Band 4 is a truncated form of catalase B, with the N terminus corresponding to residue 334 from the full-length protein sequence (determined by Edman degradation analysis). The protein in band 5 appears to be Asp f2, containing the same N-terminal as full-length Asp f2. It may appear at a lower apparent molecular weight due to an altered glycosylation pattern, or it may have a truncated C terminus.

Spectral comparisons of intact A. fumigatus biofilms (cells plus matrix) and the isolated ECM. To obtain quantitative detail on the A. fumigatus ECM composition, we employed a top-down

FIG 1 Electron microscopy of A. fumigatus biofilm preparations and ECM protein analysis. (A to C) Scanning electron micrographs of intact A. fumigatus biofilm. (A) This lower-magnification micrograph shows the collection of hyphae associated with the A. fumigatus biofilm formed in the modified version of RPMI medium used in this study. ECM connective material is present between some of the hyphae (indicated by red arrows). (B) At higher magnification, wide sheets of ECM are seen stretching between hyphae. (C) ECM that is closely associated with hyphae can be observed at the highest resolution, with an apparent rough and web-like coating. (D) Transmission electron micrograph of isolated A. fumigatus ECM. Fibrous material is indicated by a red arrow, and vesicle-type structures are also apparent. (E) SDS-PAGE protein gel of the A. fumigatus ECM. Five representative bands of the SDS-soluble proteins (labeled 1 to 5) were excited and identified. Catalase B (band 1, 79,910 Da) and Asp f2 (band 2, 32,838 Da) were specifically identified. Bands 3 to 5 corresponded to processed forms of these two proteins.

125.52 MHz for $^{13}$C, and 50.58 MHz for $^{15}$N) and an 89-mm wide-bore Agilent magnet at 11.7 T (500.92 MHz for $^1$H, 125.97 MHz for $^{13}$C, 50.76 MHz for $^{15}$N, and 202.78 MHz for $^{31}$P), Varian/Agilent consoles, and home-built four-frequency transmission line probes with a 13.66-mm-long, 6-mm-inner-diameter sample coil and a Revolution NMR magic-angle spinning (MAS) Vespel stator. Samples were spun in thin-wall 5-mm-outer-diameter zirconia rotors (Revolution NMR, LLC) at 7,143 ± 2 Hz, using a Varian MAS control unit. The temperature was maintained at −10°C. For all NMR experiments, π pulse lengths were 7 μs for $^1$H and 10 μs for $^{13}$C, $^{15}$N, and $^{31}$P. Proton-carbon and proton-nitrogen cross-polarization occurred at 50 kHz for 1.5 ms unless otherwise noted. Proton dipolar coupling was measured at 90 kHz with two-pulse phase modulation (TPPM). For all experiments, the recycle delay was 2 s. The $^{13}$C, spectra were referenced to tetramethylsilane (TMS), $^{15}$N spectra were referenced to phosphoric acid. All chemical shift references were determined relative to an adamantane standard. Rotational-echo double-resonance (REDOR) dephasing was calculated using peak integral values. Multipeak fitting and integration were performed in software written for Igor Pro (WaveMetrics, Lake Oswego, OR, USA).
solid-state NMR approach, as described in the introduction, which does not depend upon sample solubility or crystallinity. A standard way to obtain a carbon spectrum by solid-state NMR spectroscopy is through cross-polarization magic-angle spinning (CPMAS), introduced in 1976 (27, 28). During CPMAS, carbon magnetization is enhanced through spin polarization contact with an abundant 1H spin lattice, which is present in nearly all biomaterials (28), and the sample is mechanically spun at the magic angle to help suppress dipolar couplings and chemical shift anisotropy that would broaden the resonances significantly. The 13C CPMAS spectrum of the intact A. fumigatus biofilm containing hyphae plus associated ECM has strong polysaccharide peaks (anomerics, 100 to 105 ppm; ring-sugar carbons, 60 to 85 ppm), consistent with the thick hyphal cell walls (45,000 scans). The 13C CPMAS spectrum of the ECM is consistent with a material containing proteins (carbonyl and alpha carbon peaks), aromatic compounds (such as melanin), and polysaccharides (48,000 scans). The spinning side bands, which are a result of the magic-angle spinning and not representative of a specific carbon type, are indicated by asterisks. The 15N CPMAS spectrum of the whole biofilm contains a prominent peak centered at 122 ppm that is indicative of amides. The smaller peaks are indicative of nitrogen-containing amino acid side chains (12,000 scans). The 15N CPMAS spectrum of the ECM contains a similarly prominent amide peak centered at 119 ppm plus other smaller peaks and nitrogen contributions (12,000 scans).

The differences between the spectra of the biofilm and extracted ECM, together with the protein gel and EM characterization of the ECM, suggest that the ECM extraction resulted in a material free of hyphal contamination.

To use peak integrals or peak heights from CPMAS spectra quantitatively, one must account for the possibility that some carbons might exhibit differences in cross-polarization efficiency or relaxation. Thus, CPMAS spectra were obtained as a function of CP time, where normalized intensities could be obtained based on possible differences due to CP (see Fig. S2 in the supplemental material). Nearly all carbons exhibited similar CP behavior, with differences noted in some aliphatic carbons. The polysaccharide anemic carbons and associated ring sugar carbons are a prominent spectral feature and account for approximately 43% of the total carbon mass. The relative contributions of other spectral regions within the 13C spectrum, after all the carbon pools were first more fully annotated using additional NMR measurements described below, are fully outlined in Table 1.

To assess possible compositional variation between different biofilm samples and ECM extractions, we collected 13C CPMAS spectra of two separately cultured biofilm samples and their corresponding identically prepared ECM samples (Fig. 3). The major differences between samples obtained from the two separate cultures occur in peaks uniquely attributed to polysaccharides, and changes in overall polysaccharide content in the intact biofilms were similarly present in the corresponding extracted ECM preparations. As anticipated, ECM extracted from biofilms with high overall polysaccharide content also had greater polysaccharide content. In the ECM samples, the percentage of carbon that is attributed to polysaccharides ranges from 20 to 43% of the total carbon mass. Therefore, differences in ECM composition could be attributed to biological variability in biofilm production and not necessarily to differences in ECM extraction. The fact that the polysaccharide carbons increase without associated increases in carbonyls or α-carbons indicates that these polysaccharides are not significantly modified, unlike the more complex Vibrio polysaccharide (VPS), which contains extensive acetyl and glycine modifications (21). The observed variability in the A. fumigatus ECM polysaccharide content is in contrast to what we have previously observed for Escherichia coli ECM, which maintains a nearly constant protein-to-polysaccharide ratio of 6:1 for samples grown in the same way (20). Thus, we speculate that there may be more flexibility in the range of polysaccharide required to permit biofilm formation in biofilms with relatively high polysaccharide content. All subsequent NMR experiments and analyses were performed on the biofilm and corresponding ECM samples with greater polysaccharide content.

The 15N CPMAS spectrum of A. fumigatus biofilm contains a prominent peak centered at 122 ppm as well as peaks corresponding to multiple amine types and amino acid side chains (Fig. 2). In contrast, the 15N CPMAS spectrum of the ECM contains a single prominent amide signal centered at 119 ppm, which is upfield of the amide peak in the biofilm spectrum, encompassing 90% of the total integrated spectral area (Fig. 2). The quantitative 15N spectral contributions were also determined by analysis of CPMAS spectra obtained as a function of CP time (see Fig. S5 in the supplemental material). Amides are present in all peptides and some amino acid side chains and can be present as modifications among other biomolecules, e.g., N-acetyl modifications. The 15N peaks at 34...
and 40 ppm correspond to amines, such as those present in the side chain of lysine and those present as amino modifications of other biomolecules. Together, the amines in the ECM account for 4% of the nitrogen spectral area. The $^{15}$N peaks at 73 and 84 ppm correspond to the side chain nitrogens of arginine and together account for 4% of the spectral area of the ECM. The spectrum also contains a peak at 174 ppm, where imidazole nitrogens in histidine side chains would appear, and accounts for 2% of the ECM spectrum.

**TABLE 1**

Defined carbon pools in the *A. fumigatus* ECM (grown in modified RPMI medium)

<table>
<thead>
<tr>
<th>Chemical shift, $^{13}$C (ppm)</th>
<th>Carbon type</th>
<th>Carbon mass (% of total)</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>173</td>
<td>Carbonyls</td>
<td>12</td>
<td>Amino acids, phospholipids, N- and O-acetyl modifications</td>
</tr>
<tr>
<td>130–160</td>
<td>Aromatics</td>
<td>3</td>
<td>Melanin, amino acids</td>
</tr>
<tr>
<td>94–104</td>
<td>Anomerics</td>
<td>8*</td>
<td>Sugars</td>
</tr>
<tr>
<td>60–80</td>
<td>Ring carbons</td>
<td>35*</td>
<td>Sugars</td>
</tr>
<tr>
<td>50–60</td>
<td>Other C$_\alpha$</td>
<td>7</td>
<td>Amino acids</td>
</tr>
<tr>
<td>38–43</td>
<td>Gly C$_\alpha$</td>
<td>0.30</td>
<td>Gly</td>
</tr>
<tr>
<td>33</td>
<td>CH$_3$s</td>
<td>10</td>
<td>Amino acids, fatty acid chains</td>
</tr>
<tr>
<td>12–25</td>
<td>Methyls</td>
<td>18</td>
<td>Acetyl groups, amino acids</td>
</tr>
</tbody>
</table>

*Values are provided for the first sample based on the full analysis in this paper. The notable difference in a second ECM sample is the reduced polysaccharide content with 4% anomeric carbons and 15% ring carbons.*

Detailed analysis of the *A. fumigatus* ECM carbon pools using $^{13}$C($^{15}$N) REDOR NMR. Protein carbonyls and $\alpha$-carbon chemical shifts appear at 173 and 50 to 60 ppm, respectively. How-
ever, carbons from other biomolecular groups may also contribute to these spectral regions. Proteins contain amino acids with α-carbons and carbonyls that are each directly bonded to nitrogen, and based upon the uniqueness of these carbon-nitrogen pairs, we are able to spectroscopically select and determine the relative amounts of nitrogen-bonded carbonyls and α-carbons within a carbon spectrum of a complex material. To quantify the relative contribution of proteins to the ECM, we employed $^{13}$C$^{15}$N REDOR with 1.6-ms dephasing to select directly bonded $^{13}$C-$^{15}$N pairs (Fig. 4, left). REDOR experiments are done in two parts: one reference spectrum is collected without dephasing pulses ($S_0$), and one “dephased” spectrum is collected with rotor-synchronized dephasing pulses ($S$) to reintroduce the dipolar couplings that are suppressed during magic-angle spinning (31). The difference spectrum ($\Delta S$) is obtained from the subtraction of the $S_0$ spectrum from the $S$ spectrum. Only carbons that are proximate to the dephasing spin contribute to intensities in the $\Delta S$ spectrum. In the case of $^{13}$C$^{15}$N REDOR with 1.6-ms dephasing, the $\Delta S$ spectrum reports on those carbons directly bonded to nitrogen. At longer dephasing times, one can extend this ruler to examine longer-range C-N contacts.

In the $^{13}$C CPMAS spectrum of A. fumigatus ECM, the carbonyl peak accounts for 12% of the carbon, and it exhibited 76% dephasing in the one-bond $^{13}$C REDOR measurement. Thus, at most, only 76% of all carbonyl carbons are directly bonded to nitrogen, which indicates that 9% of the total $^{13}$C CPMAS spectral area can be assigned to nitrogen-bonded carbonyls (76% of 12%). Carbonyl-nitrogen pairs occur in peptide bonds of proteins as well as in N-acetyl groups. Glycine α-carbons appear near 40 ppm, while all the other α-carbons contribute to the resonances centered at 55 and 60 ppm. Other chemical species can also contribute to these spectral regions, and examples include the C-6 carbons of hexose-based polysaccharides. Thus, the REDOR measurement is used to quantify the percentage of these carbons that are adjacent to a nitrogen and thus can be attributed to α-carbons. The integrals of the resonances at 55 and 60 ppm together account for 14% of the total $^{13}$C CPMAS spectral area (Fig. 2), and the region exhibited 52% dephasing. Thus, we can place an upper limit on the carbon mass that can be attributed to protein α-carbons as 7% (52% of 14%). The region near 40 ppm exhibited 30% dephasing, suggesting that 0.3% of the total carbon mass is due to glycol α-carbons. The approximate 1:1 ratio of the numbers of nitrogen-bonded carbonyls (9%) to α-carbons (7%) is consistent with that expected for protein, and the 1:23 ratio of glycine α-carbons (0.3%) to other α-carbons (7%) is also within expectations for the composition of average proteins. The slight 2% excess of nitrogen-bonded carbonyls relative to α-carbons would be consistent with the presence of nitrogen-bonded carbonyls in N-acetyl modifications.

The extent to which carbons were within a longer, approximately 2.5-Å proximity to a nitrogen was examined using $^{13}$C$^{31}$P REDOR with an evolution time of 8.95 ms (Fig. 4, right). As expected, the carbonyl and α-carbon regions exhibited additional dephasing. The CH$_2$ region centered at 33 ppm accounts for 10% of the total carbon spectral area and exhibits 16% dephasing. The CH$_2$ carbons that are spatially close to nitrogen encompass 2% (16% of 10%) of all carbons and can be accounted for by amino acid side chains, especially those that also contain side chain nitrogen, including arginine, glutamine, and lysine. The remaining intensity at 33 ppm can be attributed to the aliphatic CH$_2$ groups of lipids and accounts for 8% of the total carbon mass. Dephasing in the aliphatic regions (12 to 35 ppm) also was observed, indicative of the presence of amino acid side chains and possible N-acetyl modifications. The 12- to 25-ppm region, which includes methyl groups such as those in amino acids, acetyl modifications, and termini of fatty acids, constitutes 18% of the total $^{13}$C CPMAS spectral area and exhibits 27% dephasing, suggesting that 5% (27% of 18%) of carbons belong to side chains of amino acids such as alanine or exist as methyl groups of N-acetyl modifications. The observed 12% dephasing of the 72-ppm sugar peak further supports the presence of N-acetyl modifications of polysaccharides such as those in galactosaminogalactan (4), which was previously shown to be a component of the A. fumigatus ECM, or in the N-acetylglucosamine (GlcNAc) moieties involved in N-linked glycosylation of some A. fumigatus extracellular proteins.

$^{13}$C$^{31}$P couplings indicate the presence of phosphorus-containing biomolecules in the ECM. To examine the possibility that phosphorus-containing biomolecules contribute to A. fumigatus ECM, we performed $^{31}$P NMR experiments. The ECM $^{31}$P CPMAS spectrum (Fig. 5A) contained a broad resonance that could be fit by three peaks centered at $-7$, $-0.4$, and 2 ppm (see Fig. 5B in the supplemental material), with the corresponding side
bands separated by the MAS frequency. These \(^{31}P\) shifts could be due to a multitude of phosphorylated biomolecules, including lipids, proteins, sugars, or nucleic acids. To better evaluate possible origins of phosphorous contributions to the ECM, we performed \(^{13}C\{^{31}P\}\)REDOR with 8.95-ms dephasing to identify any carbons that were proximate to phosphorous (Fig. 5B). With this one-long-range REDOR evolution time, the measurement serves as a qualitative analysis that could identify the presence of a few C-P contacts in close proximity (within 6 Å) or many C-P contacts at longer range (>6 Å). We observed dephasing by phosphorous of the carbon peaks centered at 60, 72, and 80 ppm as well as the carbonyl peak at 173 ppm (Fig. 5B, dashed REDOR difference spectrum [\(\Delta S\)]). Biomolecules that contain phosphorus atoms that are spatially close to carbons with chemical shifts near 60 to 80 ppm include nucleic acids (phosphorylated ribose), phospholipids, phosphorylated sugars, and proteins that are phosphorylated on serine or threonine residues. The dephasing of the carbonyl peak by phosphorus is consistent with phospholipid and phosphorylated proteins \((21, 32)\). If the only phosphorus-containing molecules in the ECM were phospholipids, we would have observed dephasing of the 33-ppm peak corresponding to lipid aliphatics. Similarly, phosphorylated proteins could account for only a fraction of the total \(^{13}C\{^{31}P\}\)REDOR \(\Delta S\) spectrum, even if every threonine and serine in the identified ECM proteins, catalase B and Asp f2, was phosphorylated. Thus, it is possible that multiple types of biomolecules contribute to the phosphorus pools of \(A. fumigatus\) ECM.

**DISCUSSION**

Our integrated solid-state NMR, electron microscopy, and biochemical analyses of the composition of the \(A. fumigatus\) ECM extracted from cells grown in RPMI 1640 medium provide insight into the identification and contributions of biomolecules present in the ECM. Electron micrographs of the \(A. fumigatus\) biofilm revealed sheets of ECM that connected some, but not all, of the hyphae in the dense community of cells (Fig. 1). The ECM also served to coat some of the hyphae, giving them a rough and granular web-like appearance. Inspection by electron microscopy is invaluable in revealing biofilm architecture and in characterizing the extracted ECM but does not provide insight into chemical and molecular composition. The \(^{13}C\) NMR results defined the quantities of carbon types, including carboxyls, aromatic carbons, polysaccharide carbons (anomeric and nonanomeric), and aliphatics, present in the insoluble ECM material. The \(^{15}N\) and \(^{31}P\) NMR spectra provided additional detail regarding nitrogen and phosphorous pools in the ECM. Furthermore, by ensuring full incorporation of \(^{15}N\) labels into the sample, we were able to further refine and quantify specific types of carbons, including one-bond C-N pairs, such as those in peptide carbons and alpha carbons. The full definition of the carbon pools is summarized in Table 1 and provides the absolute atomic-level parameters of the ECM obtained directly from the NMR measurements. Assimilating these data as discussed further below, we find that the \(A. fumigatus\) ECM at the molecular level contains approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components, and at least 8% lipid. Additionally, we identified the presence of phosphorus-containing biomolecules in the ECM, although we did not specifically annotate them in this study.

Polysaccharides (anomeric and ring sugars) account for approximately 43% of the total ECM carbon mass (Fig. 2 and Table 1; see Fig. S2 in the supplemental material), although variability in polysaccharide content between separate cultures was observed (Fig. 3) and a second sample was reduced in its polysaccharide content (with sugar carbons representing 20% of the carbon mass). Thus, biologically, the \(A. fumigatus\) ECM exhibits variation in the range of polysaccharide production during biofilm formation under comparable conditions. This is unlike the case for \(E. coli\), where under given growth conditions, the insoluble ECM composition exhibits very little variability \((89 to 87\% curli protein and 13 to 15\% modified cellulose)\). The \(A. fumigatus\) NMR results are consistent with polysaccharides previously reported to be present in \(A. fumigatus\) biofilm, which include galactomannan, galactosaminogalactan, α-1,3 glucans, and monosaccharides. Galactosaminogalactan, which is a polymer of galactose and N-acetylg glucamine, plays a critical role in the adherence and virulence of \(A. fumigatus\) \((33)\), and the presence of N-acetyl modifications, such as occur in galactosaminogalactan, was supported by the \(^{13}C\{^{15}N\}\)REDOR analyses.

Protein accounts for approximately 40% of the total ECM carbon mass. This estimation stems from the absolute NMR determination that amino acid α-carbons and nitrogen-bonded carbonyls account for 7% and 9% of the carbon mass, respectively. Amino acids contain an average of 5.4 carbons, and thus, three additional carbons, on average, can be attributed to protein and would contribute broadly to the aliphatic spectral region, consistent with what is observed (Fig. 2). In addition, the \(^{15}N\) CPMAS (Fig. 2) spectrum and the \(^{13}C\{^{15}N\}\)REDOR results (Fig. 4) suggest that most nitrogen is present in protein and that there is very little nitrogen that exists as modifications to other biomolecules.

Protein gel analysis identified catalase B and Asp f2 as the major proteins in the ECM. Asp f2 has been associated with damage to the host epithelium and host inflammatory reactions during fungal colonization \((26)\). Accumulation of catalase B in the ECM of \(A. fumigatus\) biofilms has been detected by immunolabeling assays \((5, 34)\), and catalase is reported to play a critical role in other microbial biofilms, including those formed by \(Pseudomonas aeruginosa\) \((35)\). The presence of catalase B in the ECM is also consistent with its protective role against reactive oxygen species, specifically hydrogen peroxide, which microbes may encounter during infection of a host. Increased catalase production generally reduces the susceptibility of microorganisms to phagocytosis and killing \((36, 37)\). It was previously reported that the \(A. fumigatus\) ECM contained 2% (wt/wt) protein, which was determined using the colorimetric Bradford assay \((5)\). We similarly performed a bicinchoninic acid (BCA) protein assay to determine protein concentration. Using this colorimetric assay, we found the ECM to contain an apparent estimate of 18% (wt/wt) protein. We know from the NMR results that this value is an underestimate of the protein concentration and is likely due to inaccessibility of protein peptide bonds within the matrix to reduce \(Cu^{2+}\) ions in the BCA assay or due to complexation of \(Cu^{2+}\) by other components. This finding provides another example of how solution-based methods, particularly such chemical reaction assays used for proteins within a complex mixture, can fail to quantify composition in intact and insoluble materials such as the \(A. fumigatus\) ECM.

For the final accounting, according to carbon mass, an additional 3% of the carbon mass was attributed to aromatic molecules such as melanin \((130 to 160 ppm)\). The presence of melanin in \(A. fumigatus\) and other fungi is considered to be important for viru-
ence because it affects the immune response to fungal infection (38). We observe some dephasing in the aromatic carbon region in the $^{13}$C/$^{15}$N REDOR experiments that is consistent with melanin (example structure in Table 1) (39, 40), but due to the overlap of $^{13}$N chemical shifts of amides and indoles (near 120 ppm) in the $^{13}$N spectrum, it is not possible to unambiguously identify whether the indole nitrogens are specifically attributed to melanin. The remaining 14% of the $^{13}$C CPMAS spectral area contains additional carbonyls (excluding nitrogen-bonded carbonyls already accounted for) and aliphatics, with the presence of sharp CH$_2$ peaks, consistent with lipid molecules. Thus, the ECM contains at least 8% lipid due to aliphatic CH$_2$ groups and up to 14% lipid in total. The carbonyl dephasing by phosphorous in the $^{13}$C/$^{31}$P REDOR experiment (Fig. 5) also suggests that A. fumigatus ECM contains some phospholipids. There is no evidence for a prominent contribution from nucleic acids in the A. fumigatus ECM characterized here, as the $^{13}$C CPMAS spectrum contains a prominent contribution from nucleic acids in the tetrads.

Biofilm integrity, as electron microscopy revealed the anticipated elaboration of biofilm when harvested and that older, more mature biofilms would have nucleic acids present in higher concentration. The lack of nucleic acids in our samples also indicates that the fungal biofilm is not necessarily dependent on nucleic acids for structural function relationships. Thus, we determined the ECM composition of A. fumigatus to be approximately 40% protein, 43% polysaccharide, 3% aromatic compounds such as melanin, and up to 14% lipid. The ECM composition of A. fumigatus can be richer in polysaccharides than the ECM composition reported for Candida albicans, with 55% protein, 25% carbohydrate, 15% lipid, and 5% nucleic acid (24). The analysis of the Candida albicans matrix material, however, used different chemical and solution-based methods to estimate matrix parts. Carbohydrate content was determined colorimetrically using a phenol-sulfuric acid precipitation protocol, protein concentration was determined using a BCA assay, lipids were extracted in a phenol-sulfuric acid precipitation protocol, protein concentration was determined with solid-state NMR spectroscopy. J Am Chem Soc 130:12568–12569. http://dx.doi.org/10.1021/ja8039426.

In summary, our analyses provide fundamental atomic-level compositional parameters in the complex ECM needed to consider the relationship between composition and function in the A. fumigatus ECM. Future comparisons with the ECM analysis of A. fumigatus under other growth conditions and with ECM from other strains will enable us to further build such composition-function relationships.

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