Community participation in biofilm matrix assembly and function

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Biofilms of the fungus *Candida albicans* produce extracellular matrix that confers such properties as adherence and drug resistance. Our prior studies indicate that the matrix is complex, with major polysaccharide constituents being α-mannan, β-1,6 glucan, and β-1,3 glucan. Here we implement genetic, biochemical, and pharmacological approaches to unravel the contributions of these three constituents to matrix structure and function. Interference with synthesis or export of any one polysaccharide constituent altered matrix concentrations of each of the other polysaccharides. Each of these was also required for matrix function, as assessed by assays for sequestration of the antifungal drug fluconazole. These results indicate that matrix biogenesis entails coordinated delivery of the individual matrix polysaccharides. To understand whether coordination occurs at the cellular level or the community level, we asked whether matrix-defective mutant strains could be coaxed to produce functional matrix through biofilm coculture. We observed that mixed biofilms inoculated with mutants containing a disruption in each polysaccharide pathway had restored mature matrix structure, composition, and biofilm drug resistance. Our results argue that functional matrix biogenesis is coordinated extracellularly and thus reflects the cooperative actions of the biofilm community.

biofilm | matrix | *Candida* | polysaccharide | resistance

In a biofilm, microbes are afforded a stable environment protected by the substrate surface and an extracellular matrix. For pathogenic microorganisms, this protection has dire consequences: it is manifested through high-level resistance to antimicrobial drugs. Hence, biofilm-related infections are incredibly challenging to treat (1–5). Elucidation of matrix biogenesis mechanisms thus addresses an interesting biological question and an urgent medical need.

The most common hospital-acquired fungal pathogen, *Candida albicans,* frequently forms biofilms on implanted medical devices and often leads to lethal disseminated disease (6–8). The intrinsic resistance of biofilms is multifactorial but is due largely to the extracellular matrix encasing the biofilm cells (9–19). Although matrix accumulation is considered a biofilm-specific attribute, it has been unclear whether the matrix results from the constitutive shedding of cell wall materials or whether it reflects distinctive activities of biofilm cells. Early evidence for the constitutive secretion model came from the findings that the matrix polysaccharide component has a composition distinct from the cell wall and that the protein component of matrix is similar to the released proteins found in suspension culture (20, 21).

We recently carried out a comprehensive analysis of *C. albicans* extracellular matrix composition (21). We found that β-1,3 glucan, the one matrix polysaccharide that has been linked to biofilm drug resistance (22, 23), is a relatively minor matrix component. Abundant components included the polysaccharides α-mannan and β-1,6 glucan, which constituted 85% and 14% of the matrix carbohydrate fraction, respectively. Coisolation of these components indicated that they exist in a mannan–glucan complex (abbreviated MGCx). Each MGCx component has structural features not found in the cell wall. For example, matrix mannan exists as a much larger structure (up to 12,000 mannose residues) compared with that described for cell wall mannan (~150 residues) (21, 24). Also, matrix β-1,6 glucan exists as a linear chain, whereas the β-1,6 glucan of the cell wall is highly branched (25). Finally, no MGCx is evident in cells grown in suspension culture. Therefore, the MGCx and its components' structures provide evidence for a biofilm-specific contribution to matrix biogenesis.

Our identification of matrix polysaccharide constituents provides the opportunity to dissect the mechanisms by which they yield matrix structure and protection. Here, we present evidence that mannan and β-1,6 glucan in the matrix contribute to the profound drug resistance exhibited by *C. albicans* biofilms. We also show that impaired production of any matrix polysaccharide, mannan, β-1,6 glucan, or β-1,3 glucan, diminishes deposition of other polysaccharides in the matrix. When mutant strains are combined in mixed biofilms, both matrix production and drug resistance are restored. Our findings argue that extracellular matrix is assembled extracellularly and incorporates products from a diverse biofilm community to create a unified structure.

Results

Genetic Determinants of Matrix Polysaccharide Production. To elucidate functions of the newly discovered matrix polysaccharides, we identified genes that govern their production. We chose 38 candidate genes that are predicted to impact the matrix mannan or β-1,6 glucan (Table S1). Six genes were chosen because of their involvement in β-1,6 glucan synthesis, and 32 genes were chosen in accordance with the mannan structure associated with MGCx. Specifically, MGCx mannan has an α-1,6 backbone with α-1,2 mannan side chains, with small amounts of phospho-linked and terminal β-1,2 linked mannose. Homozygous deletion

Significance

***Candida albicans* is the most common fungal pathogen and frequently grows as a biofilm. These adherent communities tolerate extremely high concentrations of antifungals due in large part to the protective extracellular matrix. The present studies observe a novel reliance on multiple matrix constituents for structure and function. Furthermore, the results demonstrate how the biofilm community assembles these matrix components in the extracellular space. Our findings reveal a coordinated mechanism by which the defining trait of the biofilm lifestyle arises and identify a number of potential therapeutic targets.**

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mutants were constructed for 37 genes. After multiple attempts, the gene *MNN43* could not successfully be deleted, and therefore we used a heterozygous deletion mutant to test its function. Matrix was harvested from biofilms of each mutant and assayed for β-1,6 glucan by ELISA and mannan by gas chromatography (GC). We found that nine of the mutants had significantly lower levels of the corresponding matrix polysaccharide than the WT reference strain; reductions ranged from 21% to 86% (Fig. 1). Our results indicate that seven genes govern levels of matrix mannan (*ALG11, MNN9, MNN11, VAN1, MNN4-4, PMR1,* and *VRG4*), and two genes govern levels of matrix β-1,6 glucan (*BIG1* and *KRE5*).

Polysaccharide Interactions in Matrix Assembly. Examination of the mutant biofilms by electron microscopy yielded a striking observation: each mutant caused nearly complete elimination of extracellular matrix (Fig. 1*A*). This finding is consistent with our proposal that matrix polysaccharides are in the MGCx. To test the idea that assembly of individual matrix polysaccharide components is dependent on the others, we measured levels of all three matrix polysaccharides—β-1,6 glucan, mannan, and β-1,3 glucan—in the mutants defective in each matrix component (Fig. 1*B*). We used the mutant strains discussed above as well as a *TET-FKS1* strain, which has reduced β-1,3 glucan synthase expression and a consequent reduction in matrix β-1,3 glucan (26). We also measured levels of matrix mannan and β-1,6 glucan in previously studied mutants lacking glucan modifier enzymes (27) to determine if deficient β-1,3 glucan delivery impacted matrix carbohydrate interactions (Fig. S1). Remarkably, we detected lower levels of all polysaccharides in each tested mutant strain. Because the synthesis pathways for each carbohydrate are distinct, our findings argue that there is a physical or regulatory interaction among the three matrix components.

We used pharmacological and enzymatic approaches to provide complementary evidence for the interaction of constituents in matrix assembly. Mannan accumulation was blocked with tunicamycin (TM), an antibiotic that inhibits *N*-glycosylation, and α-mannosidase, an enzyme that catalyzes the hydrolysis of terminal mannosides. We also examined the impact of Brefeldin A (BFA), an inhibitor of anterograde transport between the ER and the Golgi, with the goal of impairing matrix deposition of both mannan and β-1,6 glucan, as these are transported through the secretory pathway (28, 29). The concentrations of agents used did not inhibit planktonic or biofilm growth or alter cellular morphology (Fig. S2). As TM had previously been shown to inhibit the initial adhesion step of biofilm formation, we grew biofilms for 6 h before treatment (30). Inhibition of matrix mannan synthesis by TM also reduced β-1,6 and β-1,3 glucan. This phenotype was similar but less pronounced with α-mannosidase treatment (Fig. 1*C*). One explanation for our observation that matrix β-1,3 glucan levels did not decrease under α-mannosidase treatment is that the low concentrations of enzyme used resulted in incomplete hydrolysis of mannan, preventing disruption of

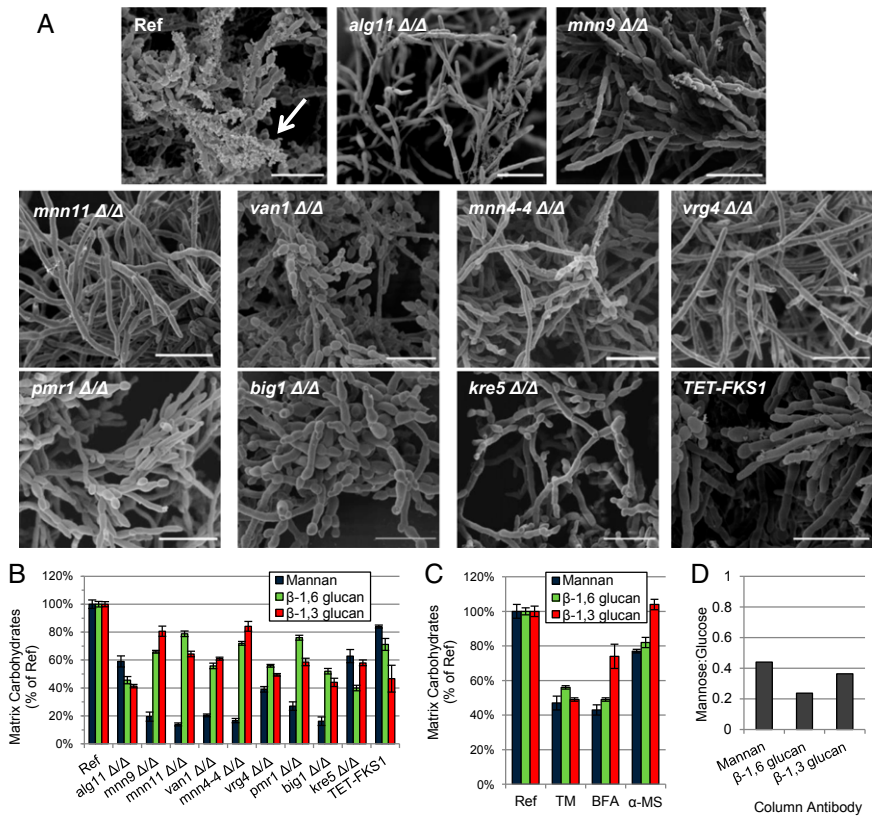

Fig. 1. Extracellular matrix polysaccharides interact and are required for matrix structure. (*A*) Biofilm morphology and extracellular matrix abundance of mutant strains and the reference strain SN250 (Ref) was assessed visually using SEM imaging. White arrow indicates extracellular matrix material. (Scale bars, 20 μm.) (*B*) Carbohydrates in the extracellular matrix of biofilms were quantified using gas chromatography analysis for mannan or ELISA with monoclonal antibodies for β-1,6 glucan and β-1,3 glucan. Data are presented as percentages of the reference strain with SEs shown. All values were significantly lower than the reference according to ANOVA ($P < 0.008$). (*C*) Carbohydrates in the matrix of WT biofilms treated with TM, BFA, and α-mannosidase (αMS) were quantified using ELISA. Data are presented as percentages of the reference strain, with mean and SEs shown. All values were significantly lower than the reference according to ANOVA, except the β-1,3 glucan concentration in α-MS–treated biofilms ($P < 0.002$). (*D*) Specific monoclonal antibodies for each matrix carbohydrate were conjugated to a CNBr-activated Sepharose 4B column. Purified extracellular matrix was run through each column, with each yielding one carbohydrate-positive fraction, which was analyzed using gas chromatography. The relative ratios of mannose to glucose were determined.

mannan–glucan interactions. The final treatment, BFA, targeting both matrix mannan and β-1,6 glucan, also resulted in reductions in matrix β-1,3 glucan. We also performed several experiments to eliminate the possibility that biofilms with treatments inhibiting the secretory pathway did not have lower levels of matrix β-1,3 glucan due to decreased production or delivery of the glucan synthase to the cell membrane. Biofilms treated with TM or BFA had normal levels of cell wall β-1,3 glucan, indicating the synthase was able to produce normal levels of glucan (Fig. S3). Additionally, treatment of planktonic cells with TM or BFA did not decrease susceptibility to an echinocandin, indicating that the drug target Fks1p was present in comparable levels. In sum, these results support the conclusion from genetic manipulations and indicate that the three matrix polysaccharides are interdependent for extracellular accumulation in the form of biofilm matrix.

A simple model to explain polysaccharide interdependence is that all three constituents, β-1,6 glucan, mannan, and β-1,3 glucan, are physically associated in biofilm matrix. To test for such interaction, we used polysaccharide-specific monoclonal antibodies (21) in crude matrix association assays. Antibody columns were loaded with crude extracellular matrix, washed, and eluted. The eluate from each specific column contained both mannose and glucose residues (Fig. 1*D* and Fig. S4). This result supports a model in which biofilm matrix has physical associations between all three carbohydrates.

Several of the genes in the current study are known to have a role in the *C. albicans* cell wall (31). Mannoproteins and β-1,6 glucan are critical components of the cell wall architecture and are linked through a glycophosphatidylinositol remnant (25, 32, 33). However, none of the genes in our investigation have previously been characterized for their role in the cell wall during biofilm growth. To characterize these structures, we used transmission electron microscopy (TEM) to image biofilm cells. On gross appearance, the outer-fibrillar layer of mannoproteins visible in the reference strain appeared reduced or absent in all strains with mannan defects (Fig. 2*A*). However, none of the mutant biofilms had significant differences in cell wall area compared with the parent strain when normalized by total cell size, suggesting the possibility of compensatory changes in individual cell wall components may allow the final structure to retain a relatively normal size (Fig. 2*B*). We also measured the cell wall carbohydrate composition of the mutant strains using gas chromatography as a complementary assay. The reference strain, SN250, contained 41% mannose and 54% glucose by dry weight, which is consistent with other reports for WT planktonic cells (25). Our results with mutant cell walls confirmed demonstrated compensatory increases in the nonmutant pathway cell wall components as has been previously described (34). Specifically, the mannan mutant strains had higher proportions of glucose, whereas strains lacking a β-1,6 glucan gene had increases in cell wall mannose (Fig. 2*C*). We observed this general trend for all of the mutants with the exception of *mnn4-4Δ/Δ* and *van1Δ/Δ*, which maintained a proportion of mannose and glucose similar to the reference strain. The finding that all mutants had either compensatory cell wall changes or no change in their carbohydrate composition stands in stark contrast to our observations with these mutants and their extracellular matrices, where every carbohydrate component was depleted. These observations suggest that disruption of these carbohydrate synthesis pathways has discrete effects on the cell wall and the extracellular matrix.

Polysaccharide Interactions in Matrix Function. We previously showed that matrix β-1,3 glucan contributes strongly to high-level fluconazole antifungal resistance of biofilms through drug sequestration (13, 35). Polysaccharide interaction is required for mature matrix structure and may be required for drug resistance as well. Therefore, we assayed susceptibility of matrix mutant biofilms to fluconazole. Each of the nine mutant strains identified by the

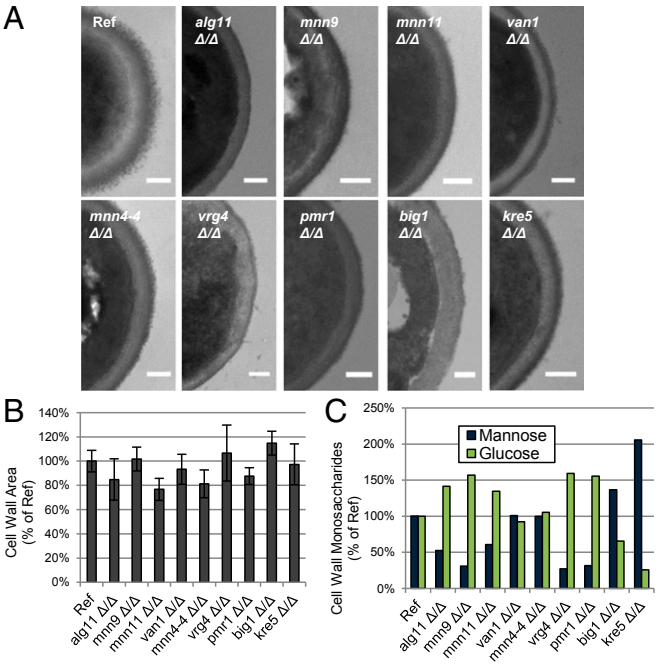

Fig. 2. Carbohydrate alterations in mutant biofilm cell walls are distinct from the extracellular matrix. (*A*) Representative images of biofilm cell wall ultrastructure, visualized using TEM. (Scale bars, 0.2 μm.) (*B*) The area of the cell wall was measured using ImageJ software. Values were normalized by the area of the total cell and are shown as a percentage of the reference strain. The mean and SEs from 10 individual cells are shown. (*C*) Cell wall carbohydrate composition was determined using gas chromatography. The percentage of the total carbohydrates in each sample comprised of mannose and glucose is shown.

matrix polysaccharide screen exhibited a profound increase in drug susceptibility, even at the relatively low concentration of 4 μg/mL fluconazole (Fig. 3*A* and Fig. S5). The susceptibility phenotype was reversed in all strains in which we could introduce a WT copy of the deleted gene. For three mutants (*van1Δ/Δ*, *mnn11Δ/Δ*, and *kre5Δ/Δ*), we were unable to isolate transformants that carried a WT gene copy, but in each case, multiple independent deletion mutant strains recapitulated the susceptibility phenotype (Fig. S6). The azole susceptibility of the deletion strains was specific to the biofilm mode of growth, as planktonic drug susceptibility was not altered for nearly all of the mutant strains. The one exception, *alg11Δ/Δ*, was slightly more susceptible to fluconazole during planktonic growth. Mutant biofilms were also susceptible to additional antifungal classes. With the exception of one strain for amphotericin B and three strains for micafungin, greater susceptibility to these drugs and 5-flucytosine was observed in comparison with the reference strain (Fig. S7). The TET-FKS1 strain was shown previously to exhibit biofilm associated susceptibility to these antifungals (36). Biofilm matrix of the WT strain was also disrupted by pharmacological and enzymatic treatments described above (TM, BFA, or α-mannosidase), and each treatment enhanced the activity of fluconazole against biofilms (Fig. 3*B*). We used radiolabeled drug (^{3}H-fluconazole) to determine directly whether matrix mannan and β-1,6 glucan are required for drug sequestration. Compared with the reference strain, each of the mannan and glucan mutant strains had a decrease in the matrix sequestration of radiolabeled drug ranging from 10% to 80% (Fig. 3*C*). These findings show that mannan, β-1,6 glucan, and β-1,3 glucan all contribute to matrix function and structure.

Extracellular Assembly of Matrix Polysaccharide. We considered two models for matrix assembly. One possibility is that matrix is

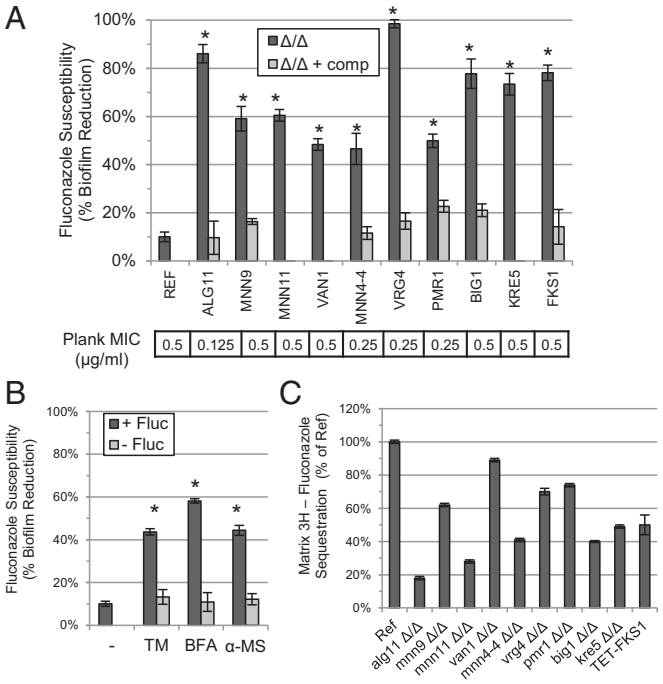

Fig. 3. Interactions of extracellular matrix carbohydrates are required for biofilm antifungal resistance. (*A*) The percent of reduction in biofilm formation following 48-h treatment with 1,000 μg/mL fluconazole compared with untreated biofilms, as quantified using the 96-well XTT assay. The null mutant (Δ/Δ) and complemented strain (Δ/Δ + comp) are shown for each gene of interest. For *FKS1*, the *TET-FKS1* strain is shown in place of a homozygous mutant, and the heterozygote strain is shown in place of a complemented strain. The minimum inhibitory concentration (MIC) of fluconazole for planktonic cells of the Δ/Δ strains is shown below. (*B*) Biofilms were treated with pharmacological inhibitors or enzymes both with and without 1,000 μg/mL fluconazole. Experiments used the same parameters as for the experiments in Fig. 1*C*, but in a 96-well plate format for quantification with the XTT assay. (*C*) Biofilms were grown for 48 h and then exposed to ^{3}H-fluconazole. Extracellular matrix was isolated for scintillation counting, and the cpm for each mutant strain was compared with the reference strain. The figure represents the mean from three technical replicates. Asterisks indicate values were significantly different from the reference strain, based on ANOVA with pairwise comparisons using the Holm–Sidak method ($P < 0.001$). SEs are shown for all panels.

assembled extracellularly, with substrates contributed by multiple cells of the biofilm community. A second possibility is that these interactions occur intracellularly before export and that biofilm properties represent only the sum total of individual cells' contributions. To distinguish between these models, we carried out an extracellular complementation assay. Specifically, we examined matrix structure and function from mixed biofilms comprised of mutant strains with defects in individual polysaccharide pathways. We reasoned that if assembly of matrix polysaccharides occurs extracellularly, then mixed mutant biofilms may produce substantial matrix to yield antifungal resistance. If assembly of matrix polysaccharides occurs intracellularly, then mixed mutant biofilms would be as defective as pure mutant biofilms. We selected one mutant strain from each carbohydrate pathway: *mnn9Δ/Δ* for mannan, *kre5Δ/Δ* for β-1,6 glucan, and *TET-FKS1* for β-1,3 glucan. All possible pairs of these mutants, as well as a triple-mixed biofilm, were assayed. We also tested mutant strains lacking glucan modifier enzymes to explore whether deficient delivery of matrix β-1,3 glucan impacts matrix carbohydrate interactions (Fig. S8). Compared with scant extracellular matrix of the individual mutant biofilms, the mixed mutant biofilms appeared similar to the reference strain by SEM imaging (Fig. 4*A*). Matrix carbohydrate analysis revealed that the deficiencies of each mutant strain were rescued in mixed biofilms (Fig. 4*B*). We also found this to be the case for mixed biofilms containing either *mnn9Δ/Δ* or *kre5Δ/Δ* mixed with a double mutant for two glucan modifier enzymes: *bgl2Δ/Δ, xog1Δ/Δ*; Fig. S8*A*). None of the mutant strain combinations formed biofilms with significantly greater biomass than the reference strain, indicating that mixing these mutants does not confer any growth advantage (Fig. S9*A*). To assay matrix function, biofilms were assayed for fluconazole susceptibility. We found that most of the mixed biofilms had similar levels of resistance to the WT reference strain (Fig. 4*C* and Fig. S8*B*). The one exception was the *kre5Δ/Δ* and *TET-FKS1* mixed biofilm; its resistance was increased slightly relative to either single mutant, but we suspect that some cross-links between β-1,6 glucan (affected by *kre5Δ/Δ*) and β-1,3 glucan (affected by

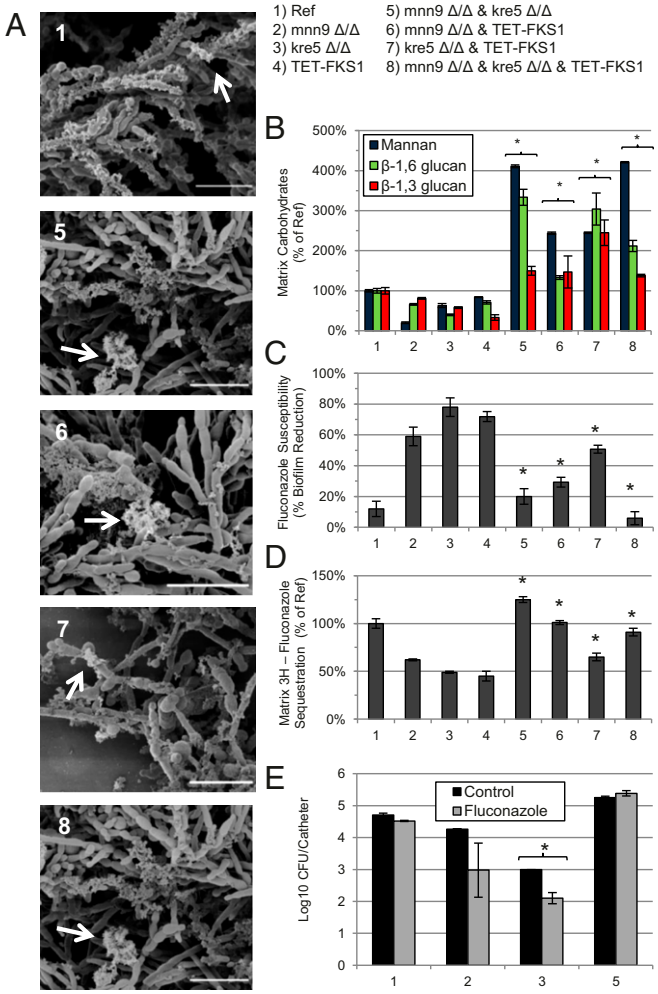

Fig. 4. Mixed mutant biofilms have restoration of extracellular matrix structure and function. Different combinations of a mannan mutant (*mnn9Δ/Δ*), a β-1,6 glucan mutant (*kre5Δ/Δ*), and a β-1,3 glucan synthase mutant (*TET-FKS1*) were used in equal number to inoculate mixed biofilms. Assays were performed as previously described: (*A*) SEM, (*B*) matrix carbohydrate content ($P < 0.05$), (*C*) biofilm reduction following fluconazole treatment ($P < 0.005$), and (*D*) matrix fluconazole sequestration ($P < 0.005$). Data for single-mutant biofilms previously presented in Fig. 1 are shown here for reference. Asterisks indicate the mixed mutant biofilm values are significantly different from their corresponding single mutant values. (*E*) The *mnn9Δ/Δ* and *kre5 Δ/Δ* mixed biofilm was tested in vivo using a rat central venous catheter model, with the effects of fluconazole or saline treatment compared with the reference and single mutant biofilms. Biofilms were quantified using viable cell counts following treatment ($P < 0.006$). Statistical analyses are based on ANOVA using pairwise comparisons with the Holm–Sidak method. Mean and SEs are shown.

TET-FKS1) may occur intracellularly. We verified increased radioactive fluconazole sequestration by representative mixed biofilm matrix preparations (Fig. 4*D*). We also tested whether a mixed biofilm could restore biofilm fluconazole susceptibility in vivo and found this to be the case for the *mnn9Δ/Δ* and *kre5Δ/Δ* combination in the clinically relevant rat central venous catheter model (Fig. 4*E*). These data demonstrate that mixed mutant biofilms regain the ability to produce functional matrix. Our observations indicate that, although some β-1,6 glucan–β-1,3 glucan assembly may occur intracellularly, the matrix polysaccharide components are capable of assembly after they have exited the cell.

Discussion

The extracellular matrix is one of the defining features of biofilm growth (17, 37, 38), providing a means for microorganisms to control their local environment. Matrix function is manifested via protection from antimicrobial therapies. This particular function of the matrix is especially relevant in the case of *C. albicans* biofilms, which form on medical devices and exhibit tolerance of antifungals of up to 1,000 times higher than those necessary to kill planktonic cells (15). In fact, current treatment guidelines recommend removal of *Candida*-infected devices, given their persistence in the face of antifungal therapy (8, 39, 40). Despite the biological and clinical significance of *C. albicans* biofilm matrix, we have a limited understanding of its biogenesis. Here we used our recent analysis of matrix constituents (21) to define genetic determinants of matrix polysaccharide synthesis. We show that each of the three major matrix polysaccharide constituents is required for assembly and function of matrix. In addition, we provide evidence that critical events in matrix assembly can occur extracellularly, and thus that matrix production represents a community activity of biofilm cells.

Two previous lines of evidence suggested that matrix biogenesis may involve a multicomponent interaction. First, in our prior investigations of β-1,3 glucan, we found that genetic disruption of this single matrix component yielded biofilms with a profound lack of extracellular material, as visualized by SEM imaging (13, 27). Second, our identification of a matrix mannan–glucan complex, MGCx, pointed to an interaction among specific matrix components that was the starting point for the current investigation (21). Specifically, we used the composition of MGCx to develop a candidate gene list for matrix biogenesis functions. We showed that many of those candidate genes were indeed required for accumulation of their expected matrix polysaccharide product. The model that polysaccharide interaction is pivotal for matrix biogenesis predicted that matrix accumulation should be dependent on each of the components, which was verified through SEM imaging and biochemical analysis. Moreover, we found that the protection from fluconazole afforded by matrix was also dependent on each polysaccharide component. These results establish that matrix structure and function both depend on multiple matrix polysaccharides.

What is the nature of the polysaccharide interaction? Given our prior identification of MGCx, and our ability to coimmunoprecipitate glucan and mannan from matrix extracts, it seems reasonable that a physical interaction among the polysaccharide components is the basis for matrix structure and function. Although further structural analysis is required, we suspect these interactions could be covalent linkages, perhaps with protein intermediates. This model of physical interaction predicts that each component-specific mutant exports the other two components, but fails to assemble them into functional matrix. The results of our mixed mutant biofilms lend support for this model, as they reveal that each mutant can provide the matrix component that is missing from a complementary mutant. Moreover, the outcome—that pathway-specific mutants participate in extracellular complementation—shows that assembly of the matrix polysaccharide complex must occur extracellularly, after export of individual components from neighboring biofilm cells. Interestingly, these mixed biofilms exhibited levels of matrix components often twofold greater than the reference strain (Fig. 4*B*). Although the reason for this is unclear, a possibility is that the abnormal matrix components act in a feedback signaling process to cause an increase in mature matrix synthesis.

Our findings provide a simple explanation for the association of matrix with the biofilm growth form of *C. albicans*. The mixed mutant biofilm experiments indicate that critical steps in matrix assembly occur extracellularly and thus that matrix assembly is a community activity. In that case, matrix assembly will be most efficient at the high cell densities that occur in a biofilm, when the substrates for assembly are at high concentrations. Viewed from this perspective, *C. albicans* matrix assembly is in essence a quorum-sensing phenomenon. The target of the secreted molecules that accumulate is not a surface receptor or response regulator but instead is the enzymes that catalyze MGCx synthesis and matrix assembly.

Materials and Methods

Please see *SI Materials and Methods* for full details.

Media. *C. albicans* strains were stored at −80 °C in 25% (vol/vol) glycerol and sustained on yeast extract-peptone-dextrose (YPD) medium with uridine. For mutant construction, transformants were selected on minimal medium with the corresponding auxotrophic supplements. Biofilms were grown in RPMI 1640 buffered with 4-Morpholinepropanesulfonic acid (MOPS), and inoculated from overnight cultures grown at 30 °C in YPD.

Strains and Strain Construction. *Candida albicans* strains were stored and grown using standard procedures. Strains used for this study are listed in Table S1. The genotypes of strains developed in the present work are shown in Table S2. The parent strain SN152 was used to create homozygous deletion strains using fusion PCR disruption cassettes as previously reported (41). Complementation of mutant strains with a single gene copy used selection for arginine prototrophy (27). Colony PCR was used to verify all genotypes; primers are listed in Table S3.

Biofilm Cell SEM. Biofilms formed on coverslips were prepared for scanning electron microscopy using osmium tetroxide and ethanol dehydration followed by critical point drying and palladium-gold coating. Samples were imaged using a SEM LEO 1530.

Biofilm Matrix and Cell Wall Collection and Analysis. Extracellular matrix and cell wall material was collected from in vitro biofilms grown in six-well plates, as published previously (27). For mixed biofilm experiments, equal volumes of the inoculum for each strain were used. For normalization of subsequent ELISA data, the mass of one biofilm from each group was assessed using crystal violet (Fig. S9*B*) (42). Biomass values for the glucan modifier strains were previously reported (27). Samples were analyzed by ELISA using biotinylated-Con A (Vector Laboratories) or monoclonal antibodies to β-1,3 glucan, β-1,6 glucan, or mannan (in house) as previously described (21, 43). GC analysis was also used to determine the concentrations and composition of monosaccharides (44).

Affinity Purification of Matrix Polysaccharides. Monoclonal antibodies for mannan, β-1,6 glucan (21, 43), or β-1,3 glucan (Biosupplies) were coupled to a CNBr-activated Sepharose 4B column (GE Life Sciences). Crude *C. albicans* extracellular matrix was loaded and eluted. Collected fractions were tested in the phenol-sulfuric carbohydrate assay (45) prior to analysis with gas chromatography or ELISA.

Biofilm Cell Wall TEM. Cells from biofilms grown in six-well plates were prepared for transmission electron microscopy and imaged as previously published (27). NIH Image J software was used to measure total cell and cell wall area of 10 cells from each strain.

Biofilm and Planktonic Cell Susceptibility to Antifungals. Biofilms in 96-well plates were tested for antifungal susceptibility as previously described (27, 36). Biofilms were quantified using a tetrazolium salt XTT reduction assay (46, 47). Biofilm reduction was calculated by comparing untreated control biofilms to those with treatment. The susceptibility of planktonically grown

C. albicans strains was assayed with the CLSI M27 A3 broth microdilution method (48). In vivo testing was performed with a previously described rat central venous catheter model (49, 50).

Sequestration of ^{3}H Fluconazole in Bbiofilms. Radiolabeled fluconazole was used in an assay to assess drug retention in biofilms formed in six-well plates (27, 51). Scintillation counting was used to measure radioactivity in the intact biofilm, extracellular matrix, cell wall, and intracellular supernatent of each tested strain.

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