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DOI: 10.1093/jac/dkz514

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Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Link to publication on Research at Birmingham portal

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Download date: 28. Feb. 2020
Candida albicans enhances meropenem tolerance of Pseudomonas aeruginosa in a dual-species biofilm

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Received 24 June 2019; returned 30 July 2019; revised 18 October 2019; accepted 12 November 2019

Background: Pseudomonas aeruginosa is an opportunistic bacterium that infects the airways of cystic fibrosis patients, surfaces of surgical and burn wounds, and indwelling medical devices. Patients are prone to secondary fungal infections, with Candida albicans being commonly co-isolated with P. aeruginosa. Both P. aeruginosa and C. albicans are able to form extensive biofilms on the surfaces of mucosa and medical devices.

Objectives: To determine whether the presence of C. albicans enhances antibiotic tolerance of P. aeruginosa in a dual-species biofilm.

Methods: Single- and dual-species biofilms were established in microtitre plates and the survival of each species was measured following treatment with clinically relevant antibiotics. Scanning electron microscopy and confocal microscopy were used to visualize biofilm structure.

Results: C. albicans enhances P. aeruginosa biofilm tolerance to meropenem at the clinically relevant concentration of 5 mg/L. This effect is specific to biofilm cultures and is dependent upon C. albicans extracellular matrix polysaccharides, mannan and glucan, with C. albicans cells deficient in glycosylation structures not enhancing P. aeruginosa tolerance to meropenem.

Conclusions: We propose that fungal mannan and glucan secreted into the extracellular matrix of P. aeruginosa/C. albicans dual-species biofilms play a central role in enhancing P. aeruginosa tolerance to meropenem, which has direct implications for the treatment of coinfected patients.

Introduction

The majority of infections in humans are polymicrobial in nature, with common diseases no longer considered to be caused by a single aetiological agent.\(^1\) The most prevalent polymicrobial infections include periodontitis, gastroenteritis, diabetic foot wounds, burn wounds and biofilm-associated infections.\(^1,2\)

The genetic disease, cystic fibrosis (CF), is characterized by thickening of the mucus layer lining the endothelium of the respiratory tract, which provides an ideal environment for microbial colonization.\(^3\) Reduced mucociliary clearance enables these microorganisms to persist and form polymicrobial biofilms on the mucosa of the lower respiratory tract.\(^1\) The CF lung is a major site of interaction between Pseudomonas aeruginosa and Candida albicans.\(^4,5\) Around 70% of CF patients become chronically infected with P. aeruginosa by the age of 30,\(^6\) with C. albicans isolated from up to 75% of CF patients,\(^7\) although sputum samples are often contaminated with microbes from the upper respiratory tract and oral cavity.\(^8\) However, simultaneous colonization has been linked to severer clinical outcomes,\(^9,10\) due to accelerated decline in lung function and worsening of disease progression.\(^10,11\)

Biofilms are structured communities of microbial cells ensnared within a matrix of extracellular polymeric substances.\(^12,13\) Biofilms are formed by bacterial and fungal species and an estimated 65%–80% of all microbial infections in humans are biofilm related.\(^2,14\) This has important clinical implications as the MICs of antimicrobials for biofilm cells can be 100–1000 times greater than for planktonic cells.\(^15,16\) Antimicrobial resistance in microorganisms poses an increasing challenge to public health worldwide,\(^17,18\) making biofilms a particularly relevant topic of research.

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Previous work on interactions between *P. aeruginosa* and *C. albicans* has focused predominantly on physical and molecular interactions and their effects on growth, morphology and virulence.27,28 However, little is known of how their interactions affect antimicrobial drug efficacy. Studies on mono-species *C. albicans* and *P. aeruginosa* biofilms have linked biofilm extracellular matrix (ECM) material to antimicrobial drug inhibition. For example, the fungal polysaccharide β-1,3-glucan sequesters the antifungal fluconazole,25 whilst the *P. aeruginosa* exopolysaccharides Pel and PsI are implicated in the inhibition of various antibiotics, including tobramycin.26 Therefore, a greater understanding of the impact of this cross-kingdom interaction on antimicrobial tolerance is of great clinical importance.

Meropenem is a first-line antibiotic for treating *Pseudomonas* infections in the CF lung.27 Meropenem is a carbapenem β-lactam that targets PBPs within Gram-negative bacteria, causing inhibition of cell wall peptidoglycan synthesis, ultimately leading to osmotic tolerance of *P. aeruginosa*28,29 due to meropenem could impede clearance of the infection. Here, we observed that *P. aeruginosa/C. albicans* dual-species biofilms displayed enhanced tolerance to meropenem. This protection was provided through active secretion of fungal ECM components, specifically mannan and β-glucan. Therefore, co-colonization of *P. aeruginosa* and *C. albicans* within the CF lung may result in small reservoirs of protected *P. aeruginosa*, which could survive antimicrobial treatment and reseed the infection site.

**Materials and methods**

**Strains and growth conditions**

Strains of *P. aeruginosa* and *Candida* species used in this study are listed in Table 1. *P. aeruginosa* strains were maintained on, and cultured in, Miller-modified LB and *C. albicans* strains in yeast extract peptone dextrose (YPD) medium. Both were grown at 37°C, with aeration at 200 rpm. Antimicrobials (from Sigma–Aldrich, UK) were used at the following concentrations (mg/L): meropenem, 0, 1, 2.5, 5 and 10; ceftazidime, 0 and 5; ciprofloxacin, 0 and 0.05; tobramycin, 0 and 2; and fluconazole, 0, 250, 500, 750 and 1000.

**Formation of dual-species biofilms**

Biofilms were grown in 96-well plates as previously described.33 Briefly, cultures were washed twice in PBS and *P. aeruginosa* cultures diluted to OD600 of 0.2 and *Candida* strains diluted to 1×106 cells/mL in Mueller–Hinton broth (MHB) or DMEM supplemented with 1% L-glutamine. *P. aeruginosa* (2×1010) and *Candida* (1×1010) cells were incubated statically in flat-bottom 96-well plates for 2 h at 37°C to enable attachment, then non-adhered cells were removed and replaced with fresh medium. After 24 h, the medium was replaced with fresh medium containing the appropriate amount of antimicrobial, or vehicle control, for an additional 18 h. To disrupt biofilms, medium was replaced with 100μL of PBS containing 50 mg/L DNase and incubated at 37°C for 1 h. Biofilms were detached using a water bath sonicator, serially diluted 1 in 10 in PBS and plated onto cetrimide agar (to determine viable *P. aeruginosa* cfu) and YPD agar supplemented with 100 mg/L tetracycline (to determine viable *Candida* cfu). Experiments were performed with three technical and at least three biological replicates.

**Formation of *P. aeruginosa* biofilms in the presence of dead *C. albicans* or ECM components**

To inactivate *C. albicans*, stationary-phase cultures were washed with PBS and cells either heat-killed at 100°C in PBS for 1 h or fixed in 1 mL of 4% parafomaldehyde (PFA) at room temperature for 1 h. Cells were then washed with PBS and diluted in MHB to 1×107 cells/mL. Subsequently, biofilms were established and quantified as above.

**Scanning electron microscopy of biofilms**

Biofilms were prepared for scanning electron microscopy using a previously published protocol34 with modifications. Single- and dual-species biofilms were grown on cell culture-treated plastic coverslips (Thermo Fisher Scientific) in 24-well plates for 24 h, after which the MHB medium was replaced with MHB with or without 5 mg/L meropenem. At 48 h, coverslips were washed twice with PBS and samples fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, at 4°C. Samples were dehydrated by increasing ethanol concentrations (50%, 70%, 90% and 100%) twice for 15 min each. Ethanol was replaced with liquid CO2 and heated up to the critical point to dry the samples. Each coverslip was mounted on a stub and sputter-coated with platinum. Scanning electron microscopy images were captured using a Philips XL30 ESEM-FEG environmental scanning electron microscope.

**Confocal microscopy of biofilms**

Single- and dual-species biofilms with or without 5 mg/L meropenem were grown as above, scaled up to a final volume of 6 mL, in 6-well plates. Medium was replaced with PBS containing 5 mg/L propidium iodide (stains dead cells), 1 μM Syto 9 (dyes DNA) and 3 mg/L calcofluor white (stains fungal cell wall chitin) and incubated at 4°C in the dark for 1 h. Biofilms were then fixed by adding 4% PFA, incubated at 4°C in the dark for 1 h and then washed twice with PBS. Confocal microscopy was performed using a Leica SP8 system equipped with a Leica DM6 upright microscope, a ×40/0.80 objective and 402, 488 and 561 nm lasers. Biofilms were imaged directly in wells with a water-dipping lens. 2-stack scans were taken at two or three different areas within each well and processed with Fiji and LASx software.

**Planktonic assay**

*P. aeruginosa* (2×1010) and *C. albicans* (1×1010) cells were added to 14 mL vent-capped culture tubes in a final volume of 2 mL MHB. Cultures were incubated for 3 h at 37°C with aeration at 200 rpm; the appropriate amount of antibiotic was added and cultures incubated for an additional 18 h. Cultures were sonicated in a water bath sonicator and serially diluted and plated for viable counts. Experiments were performed with three technical and four biological replicates.
To determine whether the presence of C. albicans increases the tolerance of P. aeruginosa to meropenem, preformed mono-species (P. aeruginosa) and dual-species (P. aeruginosa/C. albicans) biofilms were treated with meropenem and P. aeruginosa survival quantified by viable counts. Given that the concentration of meropenem in the lung immediately after administration is between 5 and 6 mg/L, this drug concentration was the focus of the study. The viability of P. aeruginosa mono-species biofilms was reduced to 25.35% when treated with 5 mg/L meropenem, indicating P. aeruginosa biofilm cells are susceptible to meropenem. Fewer P. aeruginosa cells were recovered from dual-species biofilms, which is likely due to nutrient competition. However, in the presence of C. albicans, meropenem was non-effective against P. aeruginosa in both MHB (Figure 1a) and DMEM (Figure S1, available as Supplementary data at JAC Online), indicating that this inter–kingdom interaction negatively affects meropenem efficacy.

To visualize the structure of the biofilms, samples were analysed by scanning electron microscopy and confocal microscopy. Mono-species P. aeruginosa biofilms were significantly reduced in the presence of meropenem, while in the meropenem-treated dual-species biofilms, significant levels of P. aeruginosa colonized within a C. albicans Candida enhances Pseudomonas antibiotic tolerance

Table 1. Bacterial and fungal strains used in this study

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Figure 1. *C. albicans* increases the tolerance of *P. aeruginosa* to meropenem in a dual-species biofilm. (a) Preformed 24 h biofilms were incubated for 18 h in MHB containing no antibiotic or 5 mg/L meropenem. Data are the mean ± SEM from five biological replicates. Data were analysed using two-way ANOVA and Holm-Sidak’s multiple comparisons test (**P < 0.01). NS, not significant. (b) Scanning electron microscopy analysis of biofilms. Meropenem treatment of mono-species *P. aeruginosa* biofilms results in death of bacterial cells, whilst the presence of *C. albicans* in the dual-species biofilm enhances meropenem tolerance; the tight association of *P. aeruginosa* cells to fungal surfaces is visible. *C. albicans* alone is unaffected by meropenem. (c) 3D reconstructions of biofilms from confocal z-stacks. Red indicates propidium iodide stain (dead cells), green indicates Syto 9 dye (DNA) and blue indicates calcofluor white stain (chitin).
the fungal hyphae (Figure 1b), confirming the cfu data. In agreement with this, the biofilm thickness was lower in meropenem-treated dual-species biofilms (Figure 1c and Figure S2), indicating dense packing of bacterial cells against fungal hyphae, creating a more compact biofilm structure. Therefore, the presence of C. albicans enhances the tolerance of P. aeruginosa to meropenem.

To identify whether this dual-species interaction had any impact on antifungal resistance, biofilms were treated with fluconazole. C. albicans cells in dual-species biofilms showed similar susceptibility levels to fluconazole as untreated controls at all tested concentrations. Therefore, the presence of P. aeruginosa does not affect the antifungal activity of fluconazole under the tested conditions (Figure S3).

Meropenem tolerance is not maintained following subculture of P. aeruginosa biofilm cells

The selective pressure from antibiotic use increases the likelihood of cells developing resistance. The sessile nature and close proximity of biofilm cells promotes cell–cell interactions, increasing horizontal gene transfer and mutation frequencies relative to planktonic cells. Furthermore, the presence of C. albicans increases P. aeruginosa mutation rates. To determine whether the observed increase in meropenem tolerance of P. aeruginosa was due to selection for resistance mutations, the meropenem MICs for cells recovered from both P. aeruginosa mono- and dual-species biofilms that had been treated with 5 mg/L meropenem, or untreated, were determined by standard broth microdilution assay and compared with the MIC for the starter culture. The MIC for P. aeruginosa under all tested conditions was 4 mg/L, suggesting that P. aeruginosa cells recovered from treated biofilms were not resistant to meropenem and, therefore, the observed increased tolerance was unlikely due to selection for resistance mutations.

Increased tolerance of P. aeruginosa to meropenem is specific to dual-species biofilms

Enhanced survival of P. aeruginosa as a result of interactions with C. albicans has previously been observed in planktonic cultures, through inter–kingdom communication via secreted metabolites. To determine whether the observed increase in meropenem tolerance of P. aeruginosa was specific to biofilms, P. aeruginosa susceptibility to meropenem in the presence of C. albicans was tested during planktonic growth. At all meropenem concentrations tested, there was no significant difference in P. aeruginosa survival whether in the presence or absence of C. albicans (Table 2), suggesting that C. albicans-mediated protection is biofilm specific.

Increased P. aeruginosa tolerance to meropenem is dependent on fungal viability

Production of biofilm ECM is an active process, involving secretion of glycoproteins, polysaccharides, lipids and nucleic acids. To determine whether the protective effect of C. albicans is mediated by an active or passive mechanism, P. aeruginosa biofilms were grown in the presence of either heat-killed C. albicans (disrupts the fungal cell wall, denatures proteins and causes cell lysis) or by fixing the C. albicans cells in 4% PFA (maintains cell structure). However, in the presence of heat-killed or PFA-fixed C. albicans, P. aeruginosa remained susceptible to meropenem (Figure 2), suggesting that C. albicans actively protects P. aeruginosa from meropenem.

Candida dubliensis also enhances P. aeruginosa meropenem tolerance in dual-species biofilms

Only a few species of the Candida genus are associated with disease in humans, including C. albicans, C. dubliensis, Candida tropicalis, Candida parapsilosis, Candida krusei and Candida glabrata.
The secretion of ECM polymers, specifically polysaccharides, by C. albicans biofilm cells is linked to increased antifungal resistance of fungal biofilms. However, there is increasing evidence that fungal ECM polysaccharides also contribute to antibiotic resistance in dual-species fungal/bacterial biofilms. To determine whether secreted fungal wall polymers play a role in protecting P. aeruginosa, mono-species P. aeruginosa biofilms were grown in the presence of purified fungal cell wall polysaccharides, including glucan (a mix of β-1,3-glucan and β-1,6-glucan), laminarin (an isoform of β-1,3-glucan), mannan and chitosan (deacetylated chitin). Both mannan and glucan enhanced tolerance of P. aeruginosa to meropenem in biofilms (Figure 4a), but not in planktonic cultures (Figure 4c). To determine whether mannan and glucan have independent effects, P. aeruginosa biofilms were supplemented with glucan and mannan in combination. However, no additive effect was observed (Figure 4b). Mannan and glucan protected P. aeruginosa when added to mature biofilms at the same time as meropenem (Figure 5a), suggesting that the polysaccharides may sequester or inhibit the activity of the drug. Therefore, C. albicans actively secretes mannan and/or glucan into the biofilm ECM, which protects P. aeruginosa from meropenem.

C. albicans cell wall glycosylation is important for protection against meropenem

To further investigate the role of fungal mannan in meropenem tolerance, the ability of C. albicans cell wall glycosylation mutants to protect P. aeruginosa was quantified. C. albicans has two major forms of mannan, the extensively branched N-linked mannan and the simple, linear O-linked mannan. Deletion of genes involved in key glycosylation steps results in the incorporation of altered mannan epitopes in the cell wall (Figure 5a) and within the ECM. To elucidate the role of these glycosylation structures in protecting P. aeruginosa from meropenem, mutants defective in general protein glycosylation (pmr1A, ATPase required for transporting Ca2+ and Mn2+ ions into the Golgi), N-mannan phosphomannan incorporation (mnn4A), N-mannan side chain elaboration (mnn2A, mnn2-26A) and O-mannan biosynthesis (mnt1A/mnt2A) were tested for their ability to protect P. aeruginosa from meropenem. Deletion of genes required for N-mannan biosynthesis (mnn4A, mnn2-26 and pmr1) reduced the ability of C. albicans to protect P. aeruginosa from meropenem (Figure 5b and c). Scanning electron microscopy analysis showed very few bacterial cells surviving meropenem treatment in dual-species biofilms with C. albicans glycosylation mutants (Figure 5e) but bacteria that did survive were closely adhered to the fungal cell surface, suggesting cell–cell adherence may play a role in meropenem tolerance. In agreement with this, deletion of genes involved in O-mannan biosynthesis (mnt1/mnt2), which have previously been shown to be involved in bacterial attachment to C. albicans, had the greatest impact on P. aeruginosa protection (Figure 5d and e). This indicates that protection of P. aeruginosa requires full elaboration of fungal mannan.

C. albicans does not enhance P. aeruginosa tolerance to other antibiotics

To determine whether the presence of C. albicans also affects tolerance of P. aeruginosa biofilm cells to other clinically relevant antibiotics, mono- and dual-species biofilms were treated with 5 mg/mL ceftazidime, 0.05 mg/mL ciprofloxacin, 2 mg/mL tobramycin or a combination of 5 mg/mL meropenem and 2 mg/mL tobramycin. However, the presence of C. albicans did not provide protection against these antimicrobial treatments (Figure S5a and b), suggesting that the mechanism by which C. albicans confers enhanced tolerance is likely due to the chemical structure of meropenem.

C. albicans protects P. aeruginosa CF isolates from meropenem

To explore the clinical relevance of the above findings, the ability of C. albicans to increase meropenem tolerance of clinical CF isolates was tested. Mono-species biofilms of the Midlands 1 CF isolate were tested. Mono-species biofilms of the Midlands 1 CF isolate 48 was unaffected during growth in a dual-species biofilm with C. albicans (Figure 6), suggesting that in CF patients, co-colonization with C. albicans may increase P. aeruginosa tolerance to meropenem.
Discussion

*Pseudomonas aeruginosa* and *Candida albicans* are commonly co-isolated from the sputum of CF patients, where the thickened mucus layer lining the endothelium of the lower respiratory tract provides an ideal environment for biofilm formation. Chronic *P. aeruginosa* colonization in the CF lung is correlated with increased likelihood of *C. albicans* colonization, indicating a synergistic interaction that leads to a greater decline in lung function. Here, we show that *C. albicans* significantly enhanced *P. aeruginosa* biofilm tolerance to 5 mg/L meropenem in both a laboratory strain and a CF isolate. Although the protective effect was relatively small (<1 log₁₀ change in *P. aeruginosa* cfu/mL), this may still have clinical relevance. For example, the clinical breakpoint of *P. aeruginosa* for meropenem is >8 mg/L, this increased tolerance could push the required meropenem concentration over the clinical breakpoint, categorizing the infection as meropenem resistant. This has direct implications for treatment of CF patients who present with coinfection. Standard doses of meropenem may become insufficient for treating *P. aeruginosa* infection, with combination antibiotic/antifungal therapy being a potentially more effective therapeutic option.

The protective effect of *C. albicans* was biofilm specific and dependent upon fungal ECM components. This is consistent with...
Figure 5. *C. albicans* cell wall glycosylation is important for protection against meropenem. (a) Schematic diagram representing the structure of N-mannan (including phosphomannan) and O-mannan of *C. albicans*. The points of truncation of the mutants used in this study are indicated by arrows. The pmr1Δ mutant causes loss of a Galg Ca²⁺/Mn²⁺-ATPase, affecting numerous mannosyltransferases, so the extent of truncation of the α-1,6-mannose backbone is variable. (b) N-mannan glycosylation is important for protection against meropenem. Preformed 24 h biofilms were incubated for 18 h in MHB containing no antibiotic or 5 mg/L meropenem. The N-mannan glycosylation mutants (*mnn4Δ*, pmr1Δ or *mnn2Δ*) inhibit the ability of *C. albicans* to protect *P. aeruginosa*. Tolerance to meropenem is restored in reconstituted control strains. (c) The *mnn2–26A* sextuple mutant, in which only the unsubstituted α-1,6-mannose backbone of N-mannan remains, inhibits the ability of *C. albicans* to protect *P. aeruginosa*. (d) O-mannan glycosylation is important for protection against meropenem. The mnt1Δ/mnt2Δ double mutant inhibits the ability of *C. albicans* to protect *P. aeruginosa*. Meropenem tolerance is restored when MNT1 is reconstituted. Data are the mean ± SEM from three biological replicates. Data were analysed using two-way ANOVA and Holm–Sidak’s multiple comparisons test (**P < 0.01, ***P < 0.001 and ****P < 0.0001 in panels b, c and d). NS, not significant. (e) Scanning electron microscopy analysis of biofilms. Deletion of genes required for fungal N-mannan biosynthesis (*mnn4*) or O-mannan biosynthesis (*mnt1/mnt2*) reduced the ability of *C. albicans* to protect *P. aeruginosa* from meropenem, as indicated by the reduction in the number of bacterial cells following meropenem treatment; the majority of surviving bacteria are in close contact with fungal cells. When the genes (*MNN4 or MNT1*) are reconstituted, the protective effect is restored, as evidenced by the abundance of *P. aeruginosa* cells coating the fungi in the meropenem-treated samples.
Candida enhances Pseudomonas antibiotic tolerance

Figure 6. C. albicans enhances meropenem tolerance of a P. aeruginosa CF isolate. Preformed 24 h biofilms were incubated for 18 h in MHB containing no antibiotic or 5 mg/L meropenem. Data are the mean ± SEM from five biological replicates. Data were analysed using two-way ANOVA and Holm–Sidak’s multiple comparisons test (*P < 0.05). NS, not significant.

Other reports where C. albicans ECM components have been shown to provide protection against ofloxacin and vancomycin in dual-species biofilms with Escherichia coli and Staphylococcus aureus, respectively. However, in contrast to these studies, where C. albicans ECM components provide protection against a range of antimicrobials, C. albicans ECM components only increased P. aeruginosa tolerance to meropenem. This suggests that the mechanism by which C. albicans ECM components enhance meropenem tolerance may be different to those proposed for other antibiotics. Considering the effect was not seen for other β-lactams (i.e. ceftazidime) this suggests the protective mechanism may depend on chemical structure or ability to bind mannann or β-glucan, rather than the mode of action.

Previously, mannann and β-glucan have been shown to bind and sequester antimicrobials, limiting their diffusion through biofilms. Therefore, it is possible that the actual concentration of meropenem within dual-species biofilms is significantly lower. Similar interactions have been observed in dual-species biofilms where Streptococcus mutans exopolysaccharides bind and sequester flucanazole, reducing its efficacy against C. albicans. Alternatively, mannann or glucans may coat bacterial cells, providing a physical barrier that impedes drug permeation, supporting the proposed mechanism by which S. aureus is protected from vancomycin.

Although C. albicans remains clinically the most commonly isolated Candida species, the prevalence of NAC species is increasing. C. dubliniensis was the only NAC species that protected P. aeruginosa from meropenem. This finding is of clinical relevance as, although less common than C. albicans, the prevalence of C. dubliniensis within CF patients ranges from 2.6% to 39.0% and there are cases of C. dubliniensis being co-isolated with P. aeruginosa from the lower respiratory tracts of CF patients. C. dubliniensis is the most closely related NAC species to C. albicans and, as a result, their biofilms are structurally similar, with networks of yeast and hyphal cells embedded in a comparable ECM.

Although the other NAC species produce biofilms, the composition of their ECM is considerably different and their biofilms lack hyphae, which are important for bacterial attachment. Scanning electron microscopy confirmed that most bacteria in the treated dual-species biofilms were attached to fungal hyphae, suggesting that this interaction is important for protection. This hypothesis is supported by the fact that removal of O-mannann, which is required for bacterial binding, reduced the ability of C. albicans to protect P. aeruginosa. However, given that purified carbohydrates were able to provide similar protection to C. albicans, it would suggest that ECM composition is the major contributing factor providing antimicrobial protection.

In conclusion, secreted C. albicans ECM polysaccharides protect P. aeruginosa by reducing the efficacy of meropenem. Clinically, this could result in persistent bacterial infection due to pockets of protected cells, which may then acquire true resistance as a result of continued exposure to subMIC concentrations of antibiotics. This highlights the importance of early diagnosis of dual-species biofilm infections, so that more efficacious therapeutic options, such as combination antibiotic/antifungal therapy, can be considered.

Acknowledgements

We would like to thank Paul Stanley and Theresa Morris, at the University of Birmingham’s Centre for Electron Microscopy, for assistance with scanning electron microscopy sample preparation and imaging. We thank Neil Gow, for providing the C. albicans mannann glycosylation mutant strains, and Donna MacCallum, for providing the C. krusei strain.

Funding

F.A. is supported by the Wellcome Trust Antimicrobials and Antimicrobial Resistance (AMMR) doctoral training programme (108876/Z/15/Z). Work in the laboratory of J.M.A.B. is supported by a David Phillips Fellowship to J.M.A.B. (BB/M02623X/1). Work in the laboratory of R.A.H. is supported by an MRC Career Development Award (MR/L00903X/1) and the BBSRC (BB/R00966X/1).

Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 are available as Supplementary data at JAC Online.

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