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Fungal β-1,3-Glucan Increases Ofloxacin Tolerance of *Escherichia coli* in a Polymicrobial *E. coli*/*Candida albicans* Biofilm

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In the past, biofilm-related research has focused mainly on axenic biofilms. However, in nature, biofilms are often composed of multiple species, and the resulting polymicrobial interactions influence industrially and clinically relevant outcomes such as performance and drug resistance. In this study, we show that *Escherichia coli* does not affect *Candida albicans* tolerance to amphotericin or caspofungin in an *E. coli*/*C. albicans* biofilm. In contrast, ofloxacin tolerance of *E. coli* is significantly increased in a polymicrobial *E. coli*/*C. albicans* biofilm compared to its tolerance in an axenic *E. coli* biofilm. The increased ofloxacin tolerance of *E. coli* is mainly biofilm specific, as ofloxacin tolerance of *E. coli* is less pronounced in polymicrobial *E. coli*/*C. albicans* planktonic cultures. Moreover, we found that ofloxacin tolerance of *E. coli* decreased significantly when *E. coli*/*C. albicans* biofilms were treated with matrix-degrading enzymes such as the β-1,3-glucan-degrading enzyme lyticase. In line with a role for β-1,3-glucan in mediating ofloxacin tolerance of *E. coli* in a biofilm, we found that ofloxacin tolerance of *E. coli* increased even more in *E. coli*/*C. albicans* biofilms consisting of a high-β-1,3-glucan-producing *C. albicans* mutant. In addition, exogenous addition of laminarin, a polysaccharide composed mainly of poly-β-1,3-glucan, to an *E. coli* biofilm also resulted in increased ofloxacin tolerance. All these data indicate that β-1,3-glucan from *C. albicans* increases ofloxacin tolerance of *E. coli* in an *E. coli*/*C. albicans* biofilm.

Biofilms are well-structured populations of microbial cells that are attached to a surface and embedded in a self-produced extracellular polymer matrix (1, 2). Such biofilms can be found in natural, industrial, and medical environments and can be employed for a variety of biotechnological applications (3–5). However, these structured communities also have great significance for public health, as biofilm microbial cells exhibit increased tolerance to antimicrobial agents (6). Biofilms consisting of a single pathogenic microorganism have been extensively studied in the past, and multiple processes and various structural elements have previously been implicated in axenic biofilm formation, i.e., biofilms consisting of one microbial species. For the Gram-negative bacterium *Escherichia coli*, examples include motility, virulence, surface appendages, polysaccharides, toxin-antitoxin modules, quorum sensing, and several stress responses (7, 8). In the fungal opportunistic pathogen *Candida albicans*, morphological transition, quorum sensing, adhesins, and several transcription regulators are implicated in axenic biofilm development (9–12). However, as it is becoming increasingly clear that polymicrobial biofilms are the dominant form in nature, the scientific focus is shifting toward polymicrobial biofilms. Communication, cell wall components, metabolic commensalism, and competition for nutrients and physical resources are emerging as important factors influencing the physiology of both multibacterial and bacterial-fungal biofilms (13–15). For example, interaction between *C. albicans* hyphae and *Pseudomonas aeruginosa* is mediated by candidal cell wall glucans and by factors secreted by *P. aeruginosa* such as quorum-sensing molecules and phenazines (16–19). Interestingly, studies have shown that compared to axenic biofilms, microbial cells in polymicrobial biofilms often show increased drug tolerance (20–24) and virulence (25–27).

In this study, we investigated the interplay between bacteria and fungi in a polymicrobial biofilm configuration using biofilms composed of *E. coli* and *C. albicans*, here referred to as *E. coli*/*C. albicans* biofilms. *E. coli* and *C. albicans* have been found to coexist in biofilms isolated from endotracheal tubes (28; Ilse Vandecandelaere and Tom Coenye, Ghent University, Belgium, personal communication). *E. coli* and *C. albicans* are also predominant pathogens that can grow as biofilms on urinary catheters (29). In addition, several papers describe a mutually synergistic effect in *in vivo* experiments (30–32). In this study, we first optimized a protocol to obtain *E. coli*/*C. albicans* biofilms. Next, we investigated the tolerance of these species in such an *E. coli*/*C. albicans* biofilm setup to commonly used antifungal agents with antibiofilm activity, amphotericin B and caspofungin (33), as well as to ofloxacin, a fluoroquinolone antibiotic, and kanamycin, an aminoglycoside antibiotic. We observed altered tolerance only to ofloxacin by *E. coli* in an *E. coli*/*C. albicans* biofilm compared to an axenic *E. coli* biofilm, here referred to as *E. coli* biofilm. Next, we sought to understand the molecular mechanism underlying the increased

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ofloxacin tolerance of *E. coli* in *E. coli/C. albicans* biofilms. Our results indicate that fungal β-1,3-glucan significantly influences the ofloxacin tolerance of *E. coli*.

MATERIALS AND METHODS

Strains and media. *C. albicans* strains SC5314 (34), DAY185, and CJN1201 (*zap1*Δ/*zap1*Δ) (35) and *E. coli* strain K-12 MG1655 (36) were used in this study. Overnight cultures of *C. albicans* were grown in yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, and 2% dextrose) at 30°C. Overnight cultures of *E. coli* were grown in lysogeny broth (LB) medium at 37°C. RPMI 1640 medium with L-glutamine and without sodium bicarbonate was purchased from Sigma and buffered to pH 7.0 with 3-N-morpholinepropanesulfonic acid (MOPS; final concentration, 165 mM; Sigma, St. Louis, MO). Stock solutions of ofloxacin, kanamycin, and laminarin from *Laminaria digitata* (all from Sigma, St. Louis, MO) were prepared in Milli-Q water. Tetracycline hydrochloride and amphotericin B were purchased from Sigma (St. Louis, MO), and caspofungin (Cancidas) was purchased from Merck (Beeston, Nottingham, United Kingdom). Lyticase from *Arthrobacter luteus*, proteinase K from *Tritirachium album*, N-acetylglucosaminidase from *Canavalia ensiformis*, and DNase I (all from Sigma, St. Louis, MO) were dissolved in phosphate-buffered saline (PBS).

Growth of *E. coli/C. albicans* biofilms. Overnight cultures of *C. albicans* and *E. coli* were diluted in RPMI-MOPS to optical densities at 600 nm (OD_{600}) of 0.01 and 0.001, respectively. Equal volumes of these cell suspensions were mixed, and 100 μl of this mixed cell suspension was added to the wells of a round-bottom microtiter plate (TPP, Trasadingen, Switzerland). Axenic biofilms were grown using identical inoculum densities mixed with an equal volume of RPMI. After an adhesion phase of 4 h at 37°C under nonshaking conditions, nonadherent cells were removed by rinsing with PBS. Fresh RPMI was added, and biofilms were grown for 24 h at 37°C. Afterwards, biofilms were rinsed with PBS and cells were resuspended in 100 μl PBS after sonication (1 min, 45 kHz, USC300-T; VWR, Radnor, PA, USA) and vigorous up-and-down pipetting. Dilution series were made, and quantification of *E. coli* and *C. albicans* populations was performed using selective plating, i.e., tryptic soy broth (TSB) plates containing 25 μg/ml amphotericin B and YPD plates containing 100 μg/ml tetracycline, respectively.

Visualization of biofilms. Biofilms were grown as described above, using titanium disks (Ti-6Al-4V, grade 4, annealed, 1-mm sheet thickness from GoodFellow; 15.5-mm diameter obtained by laser cutting with LaserTek) as the substrate. Afterwards, samples were washed to remove nonadherent cells by submerging samples carefully in PBS. Scanning electron microscopy (SEM) samples were prepared based on a previously published protocol (21), with some adaptations. Samples were fixed with 2.5% glutaraldehyde (2.5% glutaraldehyde in cacodylate buffer [0.1 M, pH 7.4]) for 30 min and rinsed 3 times with PBS, followed by dehydration in a series of ethanol washes (30, 50, 70, and 90% ethanol for 20 min). Then, samples were soaked 3 times in 100% ethanol for 20 min each. Following drying, samples were coated with Au-Pd using a sputtering device (Edwards S150) in order to produce a thin conductive film on the surface. A qualitative comparative analysis of the biofilms on various samples was done by SEM with an instrument (FEI XL30-FEG) operated at standard high-vacuum settings and using a 10-mm working distance and 5-keV accelerating voltage.

Antimicrobial biofilm assay. Biofilms were grown for 24 h in RPMI as described above. After rinsing with PBS, 2-fold dilution series of ofloxacin (0.39 to 3.13 μM), kanamycin (8 to 500 μM), amphotericin B (0.04 to 1.25 μM), or caspofungin (0.07 to 2.5 μM) were prepared in RPMI, added to the biofilms, and incubated for 24 h at 37°C. Afterwards, biofilm cells were quantified as described above. Survival was calculated relative to the control (vehicle treatment only; Milli-Q for ofloxacin and kanamycin and 0.5% DMSO [dimethyl sulfoxide] for amphotericin B and caspofungin).

Enzymatic degradation of the matrix. Biofilms were grown as described above. Upon growth for 24 h at 37°C, the medium was replaced with PBS containing lyticase, proteinase K, N-acetylglucosaminidase, or DNase I (50 μg/ml) for 2 h at 25°C (lyticase and N-acetylglucosaminidase) or 37°C (DNase I and proteinase K) (1, 37). PBS was used as a control. Afterwards, biofilms were rinsed and RPMI containing 0.78 μM ofloxacin was added for 24 h at 37°C. Survival of biofilm cells was determined as described above.

Survival of *C. albicans* and *E. coli* cells upon treatment with enzymes. Overnight cultures of *E. coli* and *C. albicans* were diluted to an OD_{600} of 0.01 in PBS containing 50 μg/ml lyticase, proteinase K, N-acetylglucosaminidase, or DNase and incubated for 2 h at 25°C (lyticase and N-acetylglucosaminidase) or 37°C (DNase I and proteinase K). PBS was used as a control. Survival of cells was monitored using CFU counts.

Antimicrobial biofilm assay in the presence of laminarin. An overnight culture of *E. coli* was diluted in RPMI to an OD_{600} of 0.001. The concentrations of laminarin were selected based on previous research in the lab of D. Andes (38) and were 0.25 and 0.5 mg/ml. Stock solutions of laminarin (1,000×, 250 and 500 mg/ml) were made in Milli-Q water and 50× diluted in RPMI, resulting in the 20× stocks. Afterwards, 5 μl of these 20× stocks and 95 μl RPMI containing *E. coli* cells were added to the wells of a round-bottom microtiter plate, which was incubated for 4 h on 37°C. Next, nonadherent cells were removed by rinsing with PBS, and fresh RPMI containing the appropriate amount of laminarin was added to the wells for 24 h at 37°C. Stock solutions of ofloxacin (1,000×: 0, 0.39, 0.78, 1.56, and 3.13 mM) were made and 50× diluted in RPMI. Biofilms were rinsed with PBS and RPMI with the appropriate concentrations of ofloxacin, and laminarin was added to the biofilms, which were allowed to incubate for 24 h at 37°C. Afterwards, biofilms were washed and biofilm mass was quantified using crystal violet or CFU.

Antimicrobial planktonic assay. Overnight cultures of *C. albicans* and *E. coli* were diluted in RPMI-MOPS to OD_{600}s of 0.01 and 0.001, respectively. Equal volumes of these diluted cultures were mixed and cocultured. As a control, the diluted *E. coli* culture was mixed with an equal volume of RPMI. Cultures were grown for 24 h at 37°C, washed with PBS, and subdivided into Eppendorf tubes. After centrifugation, the supernatant was removed. Stock solutions (200×) of 2-fold concentration series of ofloxacin were diluted in RPMI to their final concentration (0.09 to 0.075 μM) and added to the Eppendorf tube. Suspended cultures were transferred to glass tubes and incubated for 24 h at 37°C to mimic antimicrobial biofilm assay conditions. Afterwards, cultures were quantified as described above.

Statistical analysis. Results shown are means ± standard deviations from 3 independent biological experiments, each consisting of two technical replicates unless stated otherwise. Statistical analysis was performed using two-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison test unless stated otherwise.

RESULTS

***C. albicans* and *E. coli* form polymicrobial biofilms.** The clinically relevant RPMI medium was chosen as the growth medium for *E. coli/C. albicans* biofilms because growth rates of *C. albicans* and *E. coli* were comparable in this medium (results not shown). *E. coli/C. albicans* biofilms were grown in RPMI using inoculation OD_{600}s of 0.01 and 0.001 for *C. albicans* and *E. coli*, respectively. Visualization of *E. coli/C. albicans* biofilms, using scanning electron microscopy, showed the physical interaction between *C. albicans* and the rod-shaped *E. coli*. Both *C. albicans* hyphal and yeast cells are present in the *E. coli/C. albicans* biofilm (Fig. 1). Subsequently, we assessed whether the inoculation density of one of the species affected the density of the other species in an *E. coli/C. albicans* biofilm. Increasing inoculation densities of *E. coli* or *C. albicans* did not alter the number of CFU of *C. albicans* or *E. coli*, respectively, in an *E. coli/C. albicans* biofilm compared to the corresponding axenic biofilm, indicating that *E. coli* and *C. albi-*

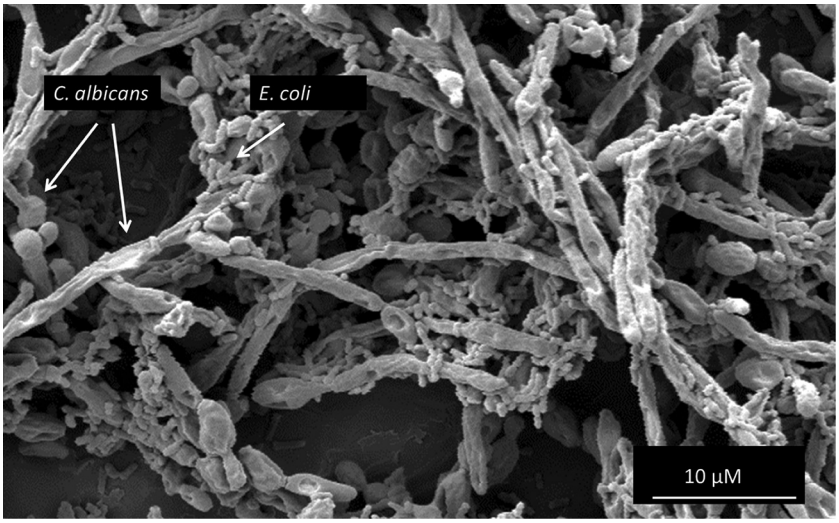

FIG 1 Interaction of *C. albicans* and *E. coli* in *E. coli/C. albicans* biofilms. *E. coli* and *C. albicans* were grown for 24 h at 37°C using titanium disks as the substrate. After dehydration, samples were visualized using SEM.

cans do not affect each other regarding their densities in the respective biofilms (see Fig. S1 in the supplemental material).

***E. coli* is less susceptible to ofloxacin in an *E. coli/C. albicans* biofilm.** Next, we determined the antimicrobial sensitivity of *E. coli* and *C. albicans* in an axenic biofilm and a polymicrobial biofilm. First, *E. coli/C. albicans* and *C. albicans* biofilms were grown in RPMI for 24 h, whereafter caspofungin or amphotericin B was added for 24 h. Survival of *C. albicans* upon treatment with caspofungin or amphotericin B in an *E. coli/C. albicans* biofilm was not significantly altered compared to survival in a *C. albicans* biofilm (see Fig. S2 in the supplemental material). Second, *E. coli/C. albicans* biofilms and *E. coli* biofilms were grown for 24 h in RPMI and subsequently treated with ofloxacin or kanamycin for 24 h. For all indicated ofloxacin concentrations, survival of *E. coli* in an *E. coli/C. albicans* biofilm was significantly increased upon ofloxacin treatment compared to survival of *E. coli* in an *E. coli* biofilm (Fig. 2), indicating that the presence of *C. albicans* reduces the efficacy of ofloxacin to act against *E. coli* biofilm cells. In contrast, we found that kanamycin tolerance of *E. coli* did not differ between an *E. coli/C. albicans* biofilm and an *E. coli* biofilm (results not shown).

Increased ofloxacin tolerance of *E. coli* is primarily biofilm specific. To test whether the observed increased ofloxacin tolerance of *E. coli* is biofilm specific, we assessed the viability of *E. coli* upon ofloxacin treatment (0.09 to 0.75 μM) under planktonic conditions, in the presence or absence of *C. albicans*. Only for one ofloxacin concentration was a significantly increased survival of *E. coli* in the presence of *C. albicans* observed compared to survival in an axenic culture (Fig. 3), suggesting that biofilm-specific characteristics, such as the extracellular matrix, are important mainly for the observed ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm.

Fungal β-1,3-glucan increases ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm. As the extracellular polymer matrix is a key component of microbial biofilms, we examined whether degradation of the extracellular matrix affects ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm. The matrix of

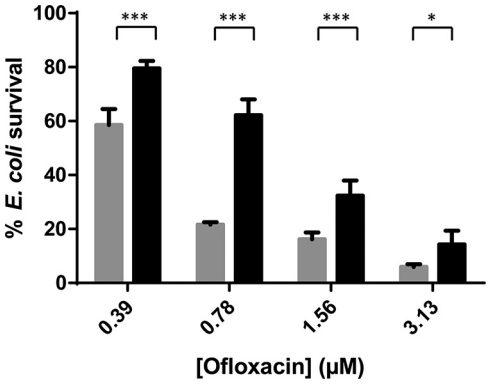

FIG 2 Increased ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm. *E. coli* (gray bars) and *E. coli/C. albicans* (black bars) biofilms were treated with different concentrations of ofloxacin (0.39 to 3.13 μM). Afterwards, survival of *E. coli* was quantified using selective plating. *, $P < 0.05$; ***, $P < 0.001$.

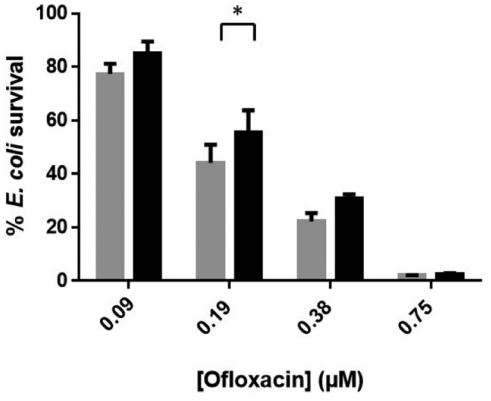

FIG 3 Increased ofloxacin tolerance of *E. coli* in the presence of *C. albicans* is mainly biofilm specific. Survival of *E. coli* in planktonic conditions upon ofloxacin treatment (0.09 to 0.75 μM) was quantified in the absence (gray bars) or presence (black bars) of *C. albicans*. *, $P < 0.05$.

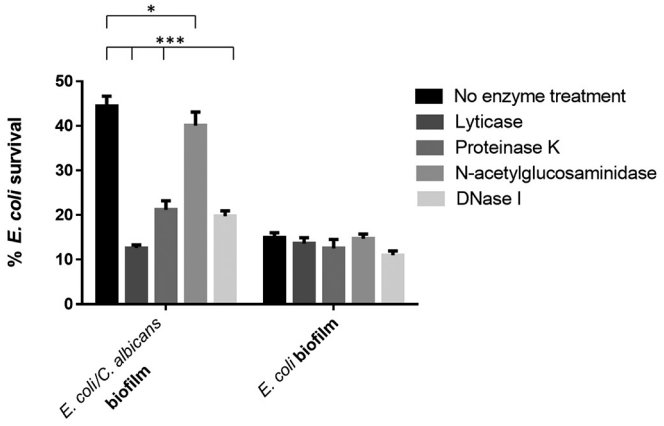

FIG 4 The extracellular matrix contributes to the observed increased ofloxacin tolerance of *E. coli* in an *E. coli*/*C. albicans* biofilm. *E. coli*/*C. albicans* and *E. coli* biofilms were treated with 0.78 μM ofloxacin with or without matrix-degrading enzymes (50 μg/ml). Afterwards, survival of *E. coli* was quantified using selective plating. *, $P < 0.05$; ***, $P < 0.001$.

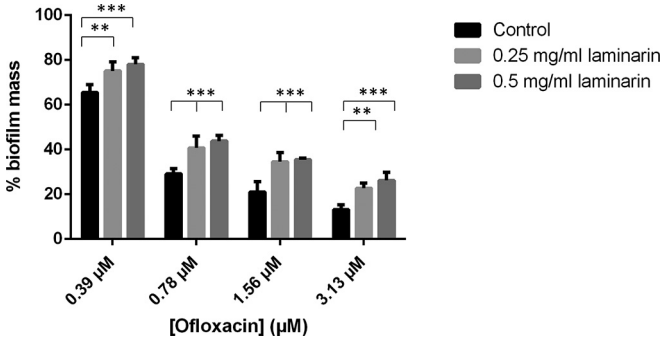

FIG 5 Exogenously added laminarin increases ofloxacin tolerance of *E. coli* in an *E. coli* biofilm. An *E. coli* biofilm was treated with different concentrations of ofloxacin in the presence or absence of different concentrations of laminarin (0 to 0.5 mg/ml). Biomass was quantified using crystal violet. **, $P < 0.01$; ***, $P < 0.001$.

C. albicans consists of proteins (55%), carbohydrates (15%), lipids (15%), and nucleic acids (5%) (39). The exopolysaccharide moiety contains α-D-glucose, β-D-glucose, α-D-mannose, α-L-rhamnose, and *N*-acetylglucosamine subunits (40). We assessed the involvement of these constituents in the observed ofloxacin tolerance by partially degrading the matrix of *E. coli*/*C. albicans* biofilms using one of the following enzymes: β-1,3-glucan-degrading lyticase, N-acetylglucosaminidase, proteinase K, or DNase I (37). Subsequently, biofilms were treated with 0.78 μM ofloxacin, as this concentration results in the most pronounced difference in *E. coli* survival between *E. coli* and *E. coli*/*C. albicans* biofilms. We found that degradation of the matrix by any of these enzymes decreased the survival of *E. coli* in an *E. coli*/*C. albicans* biofilm up to approximately 4-fold (Fig. 4). The most pronounced effect was observed with lyticase, which hydrolyzes β-1,3 glucan. Pretreatment of *E. coli*/*C. albicans* biofilms with proteinase K or DNase I resulted in a 2-fold-reduced survival of *E. coli* upon ofloxacin treatment, while pretreatment with N-acetylglucosaminidase resulted in a minor decrease in *E. coli* survival in an *E. coli*/*C. albicans* biofilm. In contrast, none of the enzymes affected survival following ofloxacin treatment of *E. coli* in an *E. coli* biofilm, indicating that these enzymes primarily degrade the fungal matrix. Note that the enzyme concentrations used did not affect survival of *E. coli* or *C. albicans* cells itself (see Fig. S3 in the supplemental material).

To confirm our hypothesis that the observed ofloxacin tolerance of *E. coli* in an *E. coli*/*C. albicans* biofilm is mainly due to the presence of β-1,3-glucan, we examined the effect of exogenously added laminarin (0 to 0.5 mg/ml) on the viability of *E. coli* biofilm cells in an *E. coli* biofilm upon ofloxacin treatment (0 to 3.13 μM). Laminarin is a polysaccharide consisting of primarily poly-β-1,3-glucan with some β-1-6- interstrand linkages and branch points. Addition of laminarin to *E. coli* biofilms results in significantly increased *E. coli* survival upon ofloxacin treatment. For example, treatment of an *E. coli* biofilm with 0.78 μM ofloxacin resulted in 29.03% ± 2.33% survival, whereas addition of 0.5 mg/ml laminarin increased *E. coli* survival up to 43% ± 2.59% (Fig. 5) when crystal violet was used as readout. In addition, results were confirmed using viable counts as a readout (see Fig. S4 in the supplemental material). In parallel, we also checked the ofloxacin tolerance of *E. coli* biofilm cells in an *E. coli*/*C. albicans zap1*Δ/*zap1*Δ biofilm. A *C. albicans zap1*Δ/*zap1*Δ strain produces 1.5- to 2-fold more β-1,3-glucan than does the wild type (35). Ofloxacin tolerance of *E. coli* in an *E. coli*/*C. albicans zap1*Δ/*zap1*Δ biofilm was significantly increased compared to ofloxacin tolerance of *E. coli* in an *E. coli*/*C. albicans* wild-type biofilm (79% ± 3.58% and 57% ± 4.75%, respectively) (Fig. 6). Note that untreated *E. coli*/*C. albicans zap1*Δ/*zap1*Δ biofilms contain 1.7-fold more *E. coli* cells than do *E. coli*/*C. albicans* wild-type biofilms ($1.5 \times 10^8 \pm 2.5 \times 10^7$ versus $8.8 \times 10^7 \pm 1.3 \times 10^7$). All these data indicate that β-1,3-glucans produced by *C. albicans* can increase ofloxacin tolerance of *E. coli*.

DISCUSSION

In the past, research focused on monospecies biofilms. However, it has become clear that most biofilms are polymicrobial in nature. The interplay between microorganisms in these biofilms might influence several characteristics of the polymicrobial biofilm, in-

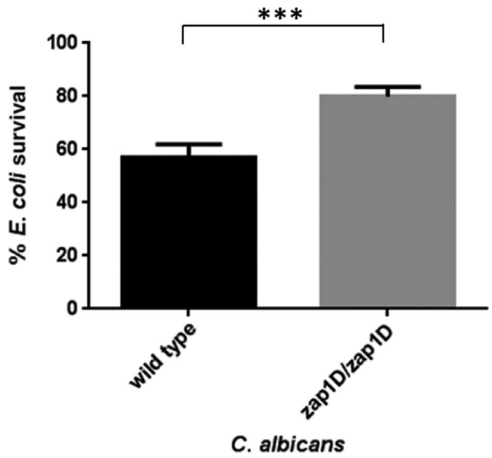

FIG 6 The presence of *C. albicans zap1*Δ/*zap1*Δ increases ofloxacin tolerance of *E. coli* to a greater extent than does the presence of *C. albicans* wild type. *E. coli*/*C. albicans* biofilms consisting of *E. coli* wild type and *C. albicans* wild type (black bars) or *zap1*Δ/*zap1*Δ deletion mutant (gray bars) were treated with 0.78 μM ofloxacin. Afterwards, survival of *E. coli* was quantified using selective plating. Statistical analysis was performed using an unpaired *t* test. ***, $P < 0.001$.

cluding drug tolerance (20–24). In this research, we compared the tolerance levels of *E. coli* and *C. albicans* to different antimicrobial agents in *E. coli/C. albicans* and monospecies biofilms. To this end, we first optimized a protocol to obtain *E. coli/C. albicans* biofilms. SEM images of the *E. coli/C. albicans* biofilm showed the physical interaction between the two species. In addition, we demonstrated that the inoculation density of *C. albicans* does not affect the density of *E. coli* cells in a 24-h-old biofilm and vice versa. Note that previously published research documented mutual antagonisms between *E. coli* and *C. albicans* in an *E. coli/C. albicans* species biofilms at defined time points (24, 41, 42). The different biofilm setup (strains, medium, incubation time, etc.) between this study and the previous studies (24, 41, 42) might account for these observed differences.

Next, the effect of *E. coli* on *C. albicans* tolerance to amphotericin B and caspofungin in an *E. coli/C. albicans* biofilm was determined. Tolerance of *C. albicans* to amphotericin B or caspofungin was not altered in an *E. coli/C. albicans* biofilm compared to an axenic biofilm. In line with our results, it was previously demonstrated that amphotericin B tolerance of *C. albicans* in a *Staphylococcus aureus/C. albicans* biofilm is not altered compared to that in a *C. albicans* biofilm (21).

In contrast to the results regarding tolerance of *C. albicans* in *E. coli/C. albicans* biofilms, we found that *E. coli* displays a significantly increased ofloxacin tolerance in an *E. coli/C. albicans* biofilm. Note that in general, oral doses of ofloxacin of 200 to 400 mg are given to patients every 12 h. Multiple-dose administration of 200-mg doses results in peak levels in serum of approximately 6 μM ofloxacin in healthy male volunteers, indicating that the ofloxacin concentrations used in this research are in line with clinically relevant concentrations. As kanamycin tolerance was not altered in an *E. coli/C. albicans* biofilm compared to an axenic *E. coli* biofilm, this indicates that the observed increased tolerance of *E. coli* in the presence of *C. albicans* is drug dependent, which is in line with recently published research of Kart and colleagues, who show that the effect of a species on the susceptibility of another species depends on the disinfectant used (43). The increased ofloxacin tolerance of *E. coli* in the presence of *C. albicans* is less pronounced under planktonic conditions, suggesting that biofilm-specific characteristics, such as the extracellular matrix, might contribute significantly to this observed increased ofloxacin tolerance. The possible contribution of matrix constituents was tested using enzymes that specifically degrade matrix components. Addition of these enzymes prior to ofloxacin treatment decreased the ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm. This effect was most pronounced using lyticase, which hydrolyzes poly-β-(1-3)-glucose such as glucan, followed by pretreatment with proteinase K and DNase. This is remarkable, as β-1,3-glucan and DNA both constitute only a small portion of the matrix (39).

Finally, we performed a detailed investigation of the effect of β-glucan on ofloxacin tolerance of *E. coli* in an *E. coli/C. albicans* biofilm using a specific *C. albicans zap1*Δ/*zap1*Δ deletion mutant that produces significantly more β-1,3-glucan than does the wild type (35) and with exogenous addition of laminarin. The polysaccharide laminarin consists primarily of poly-β-1,3-glucan with some β-1-6- interstrand linkages and branch points. First, we observed that addition of exogenous laminarin to *E. coli* biofilms increased the ofloxacin tolerance considerably. Second, our results showed that ofloxacin tolerance of *E. coli* is significantly increased in an *E. coli/C. albicans zap1*Δ/*zap1*Δ biofilm compared to an *E. coli/C. albicans* wild-type biofilm. At present, we cannot exclude that the disturbed hyphal morphogenesis of a *C. albicans zap1*Δ/*zap1*Δ biofilm, in which hyphae often end in yeast form cells, contributes to the observed phenotype (35). However, combined, these experiments indicate that β-1,3-glucan seems to play a major role in the observed increased ofloxacin tolerance of *E. coli* in *E. coli/C. albicans* biofilms.

Also in other species, β-1,3-glucan contributes to ofloxacin tolerance. For example, a *P. aeruginosa* strain with a mutation in *ndvB*, required for the formation of highly glycerol-phosphorylated β-1,3-glucans (44), displays decreased ofloxacin tolerance when grown in a biofilm compared to the wild type (45).

As β-1,3-glucan has been linked to impeded drug penetration in *C. albicans* biofilms (39, 46–48), a plausible explanation for the observed phenotype might be that β-1,3-glucan sequesters ofloxacin in an *E. coli/C. albicans* biofilm. Results of Jefferson and colleagues indicate that the increased vancomycin tolerance of *S. aureus* in a biofilm compared to its tolerance in planktonic cells could be due to a decreased penetration rate of vancomycin into the biofilm (49). Similarly, the presence of fungal β-1,3-glucan in an *E. coli/C. albicans* biofilm might account for a decreased penetration rate of ofloxacin into the biofilm, resulting in an initial exposure to a low ofloxacin concentration, which might give *E. coli* the time to initiate a defensive response.

Previous research showed that *C. albicans* also increases tolerance of *S. aureus* and *Staphylococcus epidermidis* to vancomycin (21, 22). Whereas the presence of *C. albicans* may be considered to be of minor importance in polymicrobial biofilm-associated infections, the observed increased tolerance of different Gram-positive and Gram-negative bacteria to different antibiotics, in the presence of *C. albicans*, indicates that *C. albicans* could have important clinical consequences in the treatment of these infections.

In conclusion, we show for the first time that β-1,3-glucan produced by the yeast *C. albicans* contributes to the observed increased ofloxacin tolerance of *E. coli* when grown in an *E. coli/C. albicans* biofilm. The clinical significance of these *in vitro* data will have to be determined using *in vivo* animal models.

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REFERENCES

1. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322. http://dx.doi.org/10.1126/science.284.5418.1318.
2. **Douglas LJ.** 2003. *Candida* biofilms and their role in infection. Trends Microbiol **11:**30–36. http://dx.doi.org/10.1016/S0966-842X(02)00002-1.
3. **Edwards SJ, Kjellerup BV.** 2013. Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. Appl Microbiol Biotechnol **97:**9909–9921. http://dx.doi.org/10.1007/s00253-013-5216-z.

4. **Calderon K, Gonzalez-Martinez A, Gomez-Silvan C, Osorio F, Rodelas B, Gonzalez-Lopez J.** 2013. Archaeal diversity in biofilm technologies applied to treat urban and industrial wastewater: recent advances and future prospects. Int J Mol Sci **14:**18572–18598. http://dx.doi.org/10.3390/ijms140918572.
5. **Gutierrez-Correa M, Ludena Y, Ramage G, Villena GK.** 2012. Recent advances on filamentous fungal biofilms for industrial uses. Appl Biochem Biotechnol **167:**1235–1253. http://dx.doi.org/10.1007/s12010-012-9555-5.
6. **Fanning S, Mitchell AP.** 2012. Fungal biofilms. PLoS Pathog **8:**e1002585. http://dx.doi.org/10.1371/journal.ppat.1002585.
7. **Beloin C, Roux A, Ghigo JM.** 2008. *Escherichia coli* biofilms. Curr Top Microbiol Immunol **322:**249–289.
8. **Wood TK.** 2009. Insights on *Escherichia coli* biofilm formation and inhibition from whole-transcriptome profiling. Environ Microbiol **11:**1–15. http://dx.doi.org/10.1111/j.1462-2920.2008.01768.x.
9. **Ramage G, Vandewalle K, Lopez-Ribot JL, Wickes BL.** 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. FEMS Microbiol Lett **214:**95–100. http://dx.doi.org/10.1111/j.1574-6968.2002.tb11330.x.
10. **Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG, Mitchell AP.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. PLoS Pathog **2:**e63. http://dx.doi.org/10.1371/journal.ppat.0020063.
11. **Nobile CJ, Mitchell AP.** 2005. Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. Curr Biol **15:**1150–1155. http://dx.doi.org/10.1016/j.cub.2005.05.047.
12. **Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD.** 2012. A recently evolved transcriptional network controls biofilm development in *Candida albicans*. Cell **148:**126–138. http://dx.doi.org/10.1016/j.cell.2011.10.048.
13. **Elias S, Banin E.** 2012. Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Rev **36:**990–1004. http://dx.doi.org/10.1111/j.1574-6976.2012.00325.x.
14. **Holmes AR, McNab R, Jenkinson HF.** 1996. *Candida albicans* binding to the oral bacterium *Streptococcus gordonii* involves multiple adhesin-receptor interactions. Infect Immun **64:**4680–4685.
15. **Holmes AR, Gopal PK, Jenkinson HF.** 1995. Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. Infect Immun **63:**1827–1834.
16. **Hogan DA, Vik A, Kolter R.** 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. Mol Microbiol **54:**1212–1223. http://dx.doi.org/10.1111/j.1365-2958.2004.04349.x.
17. **Brand A, Barnes JD, Mackenzie KS, Odds FC, Gow NA.** 2008. Cell wall glycans and soluble factors determine the interactions between the hyphae of *Candida albicans* and *Pseudomonas aeruginosa*. FEMS Microbiol Lett **287:**48–55. http://dx.doi.org/10.1111/j.1574-6968.2008.01301.x.
18. **Holcombe LJ, McAlester G, Munro CA, Enjalbert B, Brown AJ, Gow NA, Ding C, Butler G, O'Gara F, Morrissey JP.** 2010. *Pseudomonas aeruginosa* secreted factors impair biofilm development in *Candida albicans*. Microbiology **156:**1476–1486. http://dx.doi.org/10.1099/mic.0.037549-0.
19. **Morales DK, Jacobs NJ, Rajamani S, Krishnamurthy M, Cubillos-Ruiz JR, Hogan DA.** 2010. Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. Mol Microbiol **78:**1379–1392. http://dx.doi.org/10.1111/j.1365-2958.2010.07414.x.
20. **Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ, Kjelleberg S.** 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl Environ Microbiol **72:**3916–3923. http://dx.doi.org/10.1128/AEM.03022-05.
21. **Harriott MM, Noverr MC.** 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob Agents Chemother **53:**3914–3922. http://dx.doi.org/10.1128/AAC.00657-09.
22. **Adam B, Baillie GS, Douglas LJ.** 2002. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. J Med Microbiol **51:**344–349.
23. **Ryan RP, Fouhy Y, Garcia BF, Watt SA, Niehaus K, Yang L, Tolker-Nielsen T, Dow JM.** 2008. Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. Mol Microbiol **68:**75–86. http://dx.doi.org/10.1111/j.1365-2958.2008.06132.x.
24. **Thein ZM, Smaranayake YH, Smaranayake LP.** 2007. Dietary sugars, serum and the biocide chlorhexidine digluconate modify the population and structural dynamics of mixed *Candida albicans* and *Escherichia coli* biofilms. APMIS **115:**1241–1251. http://dx.doi.org/10.1111/j.1600-0643.2007.00735.x.
25. **Pastar I, Nusbaum AG, Gil J, Patel SB, Chen J, Valdes J, Stojadinovic O, Plano LR, Tomic-Canic M, Davis SC.** 2013. Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection. PLoS One **8:**e56846. http://dx.doi.org/10.1371/journal.pone.0056846.
26. **Peters BM, Noverr MC.** 2013. *Candida albicans-Staphylococcus aureus* polymicrobial peritonitis modulates host innate immunity. Infect Immun **81:**2178–2189. http://dx.doi.org/10.1128/IAI.00265-13.
27. **Peters BM, Jabra-Rizk MA, Scheper MA, Leid JG, Costerton JW, Shirtliff ME.** 2010. Microbial interactions and differential protein expression in *Staphylococcus aureus-Candida albicans* dual-species biofilms. FEMS Immunol Med Microbiol **59:**493–503. http://dx.doi.org/10.1111/j.1574-695X.2010.00710.x.
28. **Vandecandelaere I, Matthijs N, Nelis HJ, Depuydt P, Coenye T.** 2013. The presence of antibiotic-resistant nosocomial pathogens in endotracheal tube biofilms and corresponding surveillance cultures. Pathog Dis **69:**142–148. http://dx.doi.org/10.1111/2049-632X.12100.
29. **Samaranayake YH, Bandara HM, Cheung BP, Yau J Y, Yeung SK, Samaranayake LP.** 2014. Enteric Gram-negative bacilli suppress *Candida* biofilms on Foley urinary catheters. APMIS **122:**47–58. http://dx.doi.org/10.1111/apm.12098.
30. **Levison ME, Pitsakis PG.** 1987. Susceptibility to experimental *Candida albicans* urinary tract infection in the rat. J Infect Dis **155:**841–846. http://dx.doi.org/10.1093/infdis/155.5.841.
31. **Burd RS, Raymond CS, Dunn DL.** 1992. Endotoxin promotes synergistic lethality during concurrent *Escherichia coli* and *Candida albicans* infection. J Surg Res **52:**537–542. http://dx.doi.org/10.1016/0022-4804(92)90125-J.
32. **Klaerner HG, Uknis ME, Acton RD, Dahlberg PS, Carlone-Jambor C, Dunn DL.** 1997. *Candida albicans* and *Escherichia coli* are synergistic pathogens during experimental microbial peritonitis. J Surg Res **70:**161–165. http://dx.doi.org/10.1006/jsre.1997.5110.
33. **Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA.** 2002. Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother **46:**1773–1780. http://dx.doi.org/10.1128/AAC.46.6.1773-1780.2002.
34. **Fonzi WA, Irwin MY.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
35. **Nobile CJ, Nett JE, Hernday AD, Homann OR, Deneault JS, Nantel A, Andes DR, Johnson AD, Mitchell AP.** 2009. Biofilm matrix regulation by *Candida albicans* Zap1. PLoS Biol **7:**e1000133. http://dx.doi.org/10.1371/journal.pbio.1000133.
36. **Bachmann BJ.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol Rev **36:**525–557.
37. **Al-Fattani MA, Douglas LJ.** 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol **55:**999–1008. http://dx.doi.org/10.1099/jmm.0.46569-0.
38. **Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, VanHandel M, Andes D.** 2007. Putative role of beta-1,3 glucans in Candida albicans biofilm resistance. Antimicrob Agents Chemother **51:**510–520. http://dx.doi.org/10.1128/AAC.01056-06.
39. **Zarnowski R, Westler WM, Lacmbouh GA, Marita JM, Bothe JR, Bernhardt J, Lounes-Hadj SA, Fontaine J, Sanchez H, Hatfield RD, Ntambi JM, Nett JE, Mitchell AP, Andes DR.** 2014. Novel entries in a fungal biofilm matrix encyclopedia. mBio **5:**e01333-14. http://dx.doi.org/10.1128/mBio.01333-14.
40. **Lal P, Sharma D, Pruthi P, Pruthi V.** 2010. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. J Appl Microbiol **109:**128–136. http://dx.doi.org/10.1111/j.1365-2672.2009.04634.x.
41. **Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP.** 2009. *Escherichia coli* and its lipopolysaccharide modulate in vitro *Candida* biofilm formation. J Med Microbiol **58:**1623–1631. http://dx.doi.org/10.1099/jmm.0.012989-0.
42. **Thein ZM, Samaranayake YH, Samaranayake LP.** 2006. Effect of oral bacteria on growth and survival of Candida albicans biofilms. Arch Oral Biol **51:**672–680. http://dx.doi.org/10.1016/j.archoralbio.2006.02.005.
43. **Kart D, Tavernier S, Van AH, Nelis HJ, Coenye T.** 2014. Activity of

disinfectants against multispecies biofilms formed by Staphylococcus aureus, Candida albicans and Pseudomonas aeruginosa. Biofouling **30:**377–383. http://dx.doi.org/10.1080/08927014.2013.878333.

44. **Sadovskaya I, Vinogradov E, Li J, Hachani A, Kowalska K, Filloux A.** 2010. High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the *ndvB* gene is involved in the production of highly glycerol-phosphorylated beta-(1->3)-glucans, which bind aminoglycosides. Glycobiology **20:**895–904. http://dx.doi.org/10.1093/glycob/cwq047.
45. **Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA.** 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature **426:**306–310. http://dx.doi.org/10.1038/nature02122.
46. **Vediyappan G, Rossignol T, d'Enfert C.** 2010. Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. Antimicrob Agents Chemother **54:**2096–2111. http://dx.doi.org/10.1128/AAC.01638-09.
47. **Nett JE, Sanchez H, Cain MT, Andes DR.** 2010. Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. J Infect Dis **202:**171–175. http://dx.doi.org/10.1086/651200.
48. **Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, Hamaker J, Mitchell AP, Andes DR.** 2012. A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. PLoS Pathog **8:**e1002848. http://dx.doi.org/10.1371/journal.ppat.1002848.
49. **Jefferson KK, Goldmann DA, Pier GB.** 2005. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrob Agents Chemother **49:**2467–2473. http://dx.doi.org/10.1128/AAC.49.6.2467-2473.2005.