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Evaluation of the ability of *C. albicans* to form biofilm in the presence of phage-resistant phenotypes of *P. aeruginosa*

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Pseudomonas aeruginosa and *Candida albicans* are disparate microbial species, but both are known to be opportunistic pathogens frequently associated with nosocomial infections. The aim of this study was to provide a better understanding of the interactions between these microorganisms in dual-species biofilms. Several bacteriophage-resistant *P. aeruginosa* phenotypes have been isolated and were used in dual-species mixed-biofilm studies. Twenty-four and 48 h mixed-biofilms were formed using the isolated phenotypes of phage-resistant *P. aeruginosa* and these were compared with similar experiments using other *P. aeruginosa* strains with a defined lipopolysaccharide (LPS) deficiency based on chromosomal knockout of specific LPS biosynthetic genes. Overall, the results showed that the variants of phage-resistant *P. aeruginosa* and LPS mutants were both less effective in inhibiting the growth of *C. albicans* in mixed-biofilms compared to the wild-type strains of *P. aeruginosa*. Conversely, the proliferation of *P. aeruginosa* was not influenced by the presence of *C. albicans*. In conclusion, the ability of strains of *P. aeruginosa* to inhibit the formation of a biofilm of *C. albicans* appears to be correlated with the LPS chain lengths of phenotypes of *P. aeruginosa*, suggesting that LPS has a suppressive effect on the growth of *C. albicans*.

Keywords: lipopolysaccharides; biofilms; *C. albicans*; *P. aeruginosa*

Introduction

Bacteria and fungi co-inhabit a wide variety of environments and the interactions between them can result in substantial medical and economic impacts (Hogan & Kolter 2002; Wargo & Hogan 2006). *Pseudomonas aeruginosa*, a Gram-negative bacterium, and *Candida albicans*, a dimorphic fungus, are two important opportunistic pathogens frequently identified as the major causes of nosocomial infections (Pierce 2005; de Bentzmann & Plésiat 2011). *P. aeruginosa* is the etiologic agent for many severe nosocomial infections including bacteremia, wound infections, pneumonia, urinary tract infections, and intra-abdominal sepsis (Van Delden & Iglewski 1998; Christian van 2007; Driscoll et al. 2007; Mittal et al. 2009). *C. albicans* is known to cause a wide variety of both superficial and severe systemic diseases like infections of the skin, oral cavity and esophagus, gastrointestinal tract, urogenital and vascular systems (Molero et al. 1998; Calderone & Fonzi 2001). Co-infection by *P. aeruginosa* and *C. albicans* is not uncommon, particularly in the cases of respiratory tract infections in critically ill patients who are using mechanical ventilation devices. These infections are frequently associated with the formation of a microbial biofilm inside the endotracheal tubes (Nseir & Ader 2009). Recent studies revealed pathogenic interactions between the two microorganisms, whereby the morphology and virulence of *C. albicans* were significantly affected by the presence of *P. aeruginosa*. A quorum-sensing molecule, 3-oxo-C12 homoserine lactone, produced by *P. aeruginosa*, apparently influences the development of the filamentous form of *C. albicans*. Intriguingly, while *P. aeruginosa* forms a dense biofilm on filamentous *C. albicans* that leads to the killing of the fungus, this bacterial species neither binds to nor kills yeast-forms of *C. albicans* (Hogan & Kolter 2002; Hogan et al. 2004; McAlester et al. 2008; Nseir & Ader 2009).

Bacteriophages (phages) are viruses that specifically infect and lyse bacteria. Frequently, during bacterial infection, there is an increase in phage-resistant mutants and, since the lipopolysaccharides (LPS) are frequently identified as phage receptors in a bacterial host, phage resistance can be linked to changes in the chain length of LPS (Filippov et al. 2011). It has also been reported that the LPS from various bacterial species exerted variable effects on the inhibition of biofilms of *C. albicans* (Bandara et al. 2009; Bandara, Lam, et al. 2010; Bandara et al. 2013), indicating that LPS plays a critical role in suppressing the proliferation of the fungal pathogen in biofilm communities of mixed species.

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The inhibition of biofilms of *C. albicans* due to the presence of *P. aeruginosa* is not clearly defined. The present work aimed to provide a better understanding of the interaction between *P. aeruginosa* and *C. albicans* in dual-species biofilms and, therefore, phage-resistant phenotypes of *P. aeruginosa* and LPS mutants were used to evaluate their influence on the ability of *C. albicans* to form biofilms.

Materials and methods

Bacteria, fungi, bacteriophage and culture conditions

The *Pseudomonas* and *Candida* strains used in this work are listed in Table 1. All strains were grown at 37 °C in Yeast Peptone Dextrose (YPD) medium, which is composed of 10 g l^{-1} of yeast extract (Merck), 20 g l^{-1} of peptone (Merck) and 20 g l^{-1} of dextrose (Merck). Solid YPD medium also contained 1.2% w/v of agar (Merck) and the soft agar top-layer contained 0.6% w/v of agar. Bacteriophages, phiIBB-PAA2 and phiIBB-PAP21, were isolated from hospital effluents provided by Hospital de São João (Porto, Portugal) (Pires et al. 2011). All procedures related to the isolation and production of phages were performed with Tryptic Soy Broth (Merck) or Tryptic Soy Agar (TSA, Merck). To obtain lysates from bacterial cultures, sonication using a medium-sized probe (Ultrasonic Processor) was performed for 5 min at 40 W.

Production and concentration of bacteriophage

The phages were produced in 250 ml T-flasks and the phage lysates were concentrated and purified according to the protocol described by Sambrook and Russell (2001). Briefly, a top-agar layer, containing 3 ml of the phage solution and 3 ml of the bacterial culture grown overnight in 90 ml of soft agar, was added to T-flasks with a thin layer of TSA. After solidification, the T-flasks were incubated at 37 °C overnight. Following incubation, 90 ml of SM Buffer (5.8 g l^{-1} NaCl, 2 g l^{-1} MgSO4.7H2O, 50 ml l^{-1} 1 M Tris–HCl pH 7.5) were added to the T-flasks and incubated, under shaking conditions at 5 °C for a minimum of 5 h. The lysate was concentrated with PEG 8000 and then purified with chloroform. The phage solutions in SM Buffer were stored at 4 °C.

PFU titration of the stock solution

Plaque forming unit (PFU) counting was performed according to the double agar overlay technique (Kropinski et al. 2008). Briefly, 100 µl of diluted phage solution, 100 µl of bacterial culture, and 3 ml of soft agar were mixed into a Petri plate containing a thin layer of TSA. The plates were incubated overnight at 37 °C and then the PFUs were counted.

Formation of biofilms

Biofilms of single species were formed on 24-well microplates containing 1 ml of YPD medium and 10 µl of cellular suspension with an optical density at 600 nm (OD_{600}) of 1.0, which corresponds to ~1.9×10^9 CFU ml^{-1} for *P. aeruginosa* ATCC 10145, 1.1×10^9 CFU ml^{-1} for *P. aeruginosa* PAO1 and 1.1×10^7 CFU ml^{-1} for *C. albicans*

Table 1. *P. aeruginosa* and *C. albicans* strains used in this study.

P. aeruginosa and *C. albicans* strains	Properties and remarks	Source/reference
C. albicans CECT 1472		Colección Española de Cultivos Tipo
P. aeruginosa ATCC 10145	(A+B+ LPS phenotype) wild-type strain, serotype O6	American Type of Culture Collection
P. aeruginosa PAO1	(A+B+ in LPS phenotype) wild-type strain, serotype O5	University of Guelph, Canada
P. aeruginosa ATCC 10145_M1 to *P. aeruginosa* ATCC10145_M6	Isolates from 24 h of mixed biofilms infection with phage phiBB-PAA2	This study
Knockout isogenic LPS mutants derived from *P. aeruginosa* PAO1:		University of Guelph, Canada
(1) *wbpL* Mutant	(A−B−), deficient in the initial glycosyltransferase affecting both B-band and A-band	Rocchetta, Burrows, et al. (1998)
(2) *rmlC* Mutant	(A−B−), defective in TDP-L-rhamnose biosynthesis, with truncated outer core	Rahim et al. (2000)
(3) *wzy* Mutant	(A+B−), deficient in O-antigen polymerase for B-band biosynthesis, produces core-plus-one O-repeat unit	de Kievit et al. (1995)
(4) *rmd* Mutant	(A−B+), deficient in GDP-D-rhamnose biosynthesis becomes A-band minus, not affecting B-band	Rocchetta, Pacan, et al. (1998)

CECT 1472. Biofilms dual species were also formed in 24-microtitre plates in which the wells, containing 1 ml of YPD medium, were inoculated with 10 µl of each cellular suspension (OD_{600} of 1.0). Both biofilms of single and dual species were formed for 24 and 48 h with the renewal of medium every 12 h. The plates were incubated at 37 °C with shaking at 120 rpm in an orbital shaker.

Crystal violet assay

For characterization of biofilms, the total biomass attached to each well was measured using the crystal violet (CV) assay as previously described (Silva et al. 2009; Pires et al. 2011). Briefly, after the wells were washed with a saline solution (0.9% NaCl (Merck) in distilled water), methanol (Merck) fixation of the biomass of each well was done, followed by CV (1% v/v in water, Merck) staining and acetic acid (33% v/v, Merck) elution. The eluted stain aliquots from each well were placed in a 96-well microtiter plate and absorbance at 570 nm (A_{570}) was determined by using an ELISA plate reader.

XTT reduction assay

Quantification of the cellular activity of the biofilm was measured through a reduction assay with 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT) using a previously described method (Silva et al. 2010; Pires et al. 2011). The wells of a 24-well microtiter plate were washed with saline solution (0.9% NaCl) and later 1 ml of XTT solution (200 mg l^{-1}, Sigma) containing PMS (20 mg l^{-1}, Sigma) was added into each well. The plates were incubated at 37 °C for 3 h in the dark and then the absorbance at 490 nm (A_{490}) was read with an ELISA plate reader in 96-well microtiter plates.

Scanning electron microscopy of the biofilms

Biofilms were also analyzed by scanning electron microscopy (SEM; S-360, Leo, Cambridge, USA) following standard preparative techniques. Briefly, biofilms formed on slides cut from the bottom of 24-well microplates were washed with saline solution, dehydrated with alcohol (20% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and were allowed to dry prior to coating with gold.

Infection of biofilms

To investigate the pattern of growth recovery of the *Candida* biofilm, once the *Pseudomonas* biofilm cells were lysed, phages specific for the *P. aeruginosa* strains to eliminate the bacterial cells from the dual-species biofilm were used. For this, dual-species biofilms (*P. aeruginosa* ATCC 10145 + *C. albicans* CECT 1472 and *P. aeruginosa* PAO1 + *C. albicans* CECT1472) were, respectively, infected with previously characterized phages, phiIBB-PAA2 (*P. aeruginosa* ATCC 10145 host) and phiIBB-PAP21 (*P. aeruginosa* PAO1 host) (Pires et al. 2011).

After biofilm formation for 24 h, all the medium and planktonic bacteria were removed from each well and the wells were washed twice with fresh YPD medium. Afterwards, 500 µl of fresh YPD medium and 500 µl of phage solution or SM buffer, in the case of control experiments, were added. The multiplicity of infection used in infection of biofilms was 1. The plates were incubated under agitation (120 rpm) at 37 °C and samples were taken after 2, 6 and 24 h infection of biofilm for counting the colony forming units (CFU) and PFUs (number of phages attached and released from biofilms).

Counts of CFU and PFU in the attached bacteria, yeast or phages

For both mono- and dual-species biofilms, the microdrop technique was used to count the CFU that correspond to the number of viable cells present in the biofilms. First, the wells of the microplates were washed twice with saline solution (0.9% NaCl) to remove unattached microorganisms and then, 1 ml of fresh saline solution was added to each well. After this, the biofilm was scraped with a scraper and the samples were taken, diluted in saline solution and one drop (10 µl) was placed into a Petri plate and allowed to run down the plate (Silva et al. 2010; Pires et al. 2011). Titers of *P. aeruginosa* were determined by plating on YPD solid medium supplemented with amphotericin B (10 µg ml^{-1}) in order to suppress fungal growth and *C. albicans* was plated on YPD solid medium supplemented with tetracycline (50 µg ml^{-1}) to suppress the growth of *P. aeruginosa*. The plates were incubated at 37 °C for 16 to 18 h and then the CFUs were counted.

To determine the number of phages attached to the biofilms, the Small Drop method with some modifications was used (Mazzocco et al. 2008). Briefly, biofilms were washed, resuspended in 1 ml of fresh saline solution and scraped as described for CFU counts. Then, samples were taken, diluted in SM buffer and 20 µl of the diluted phage solution were added to 20 µl of the host solution grown overnight and was incubated for 15–20 min in order to allow the phage to bind the host. After this, 20 µl of each sample were placed in an agar plate and allowed to dry. The plates were incubated overnight at 37 °C to allow for the PFU counts.

Preparation of lysates of P. aeruginosa

Cultures of *P. aeruginosa* ATCC 10145 were infected with phage phiIBB-PAA2 for 24 h. The culture was centrifuged (9,000 × *g*, 10 min, 4 °C), the supernatant

was collected, and the lysates were filtered (0.22 μm) to remove all non-lysed cells. The filtered lysates were applied to 24 h old biofilms of *C. albicans* for the same 24 h conditions as used in the assays for phage infection of dual-species biofilms.

Isolation of phage-resistant phenotypes of P. aeruginosa

After infection of dual-species biofilm with phage phiIBB-PAA2 for 24 h, several colonies of *P. aeruginosa* were picked and isolated. Then, the isolated colonies were tested for their susceptibility to the phages used in this study – phiIBB-PAA2 and phiIBB-PAP21. In total, six phenotype variants, named as *P. aeruginosa* ATCC_M1 to *P. aeruginosa* ATCC_M6 were isolated (these strains are described as M1–M6).

Preparation of LPS

The samples of bacterial LPS were prepared according to the protocol described by Hitchcock and Brown (H&B) (Hitchcock & Brown 1983). Briefly, 1 ml of a bacterial culture at OD_{600} of 0.45 was centrifuged (1 min, 10,000 × *g*) and the pellet was suspended in 250 μl of the lysis buffer (2% w/v SDS, 4% v/v 2-mercaptoethanol, 10% v/v glycerol, 1.0 M Tris–HCl pH 6.8, bromophenol blue for colour). The lysates were heated at 100 °C for 30 min, 1.5 μl of 20 mg ml^{-1} proteinase K (Sigma) were added to them, they were incubated overnight at room temperature, and centrifuged for 1 min at 10,000 × *g*.

SDS-PAGE Western immunoblotting of bacterial LPS

Acrylamide running gels at 12% were prepared according to a modified Laemmli procedure with resolving gels devoid of SDS (Laemmli 1970). Three μl samples of H&B LPS, except for the O6 B-band blot in which the sample used was only 1 μl, were loaded on the acrylamide gel and run at 150 V for 50 min. Blotting was performed for 60 min at 200 mA. Skimmed milk (5%) in PBS was used to block the nitrocellulose (NC) blots (Rocchetta & Lam 1997). Primary monoclonal antibodies (mAb) specific against *P. aeruginosa* LPS were from cell lines of mouse hybridoma, and the supernatants of the culture of these were used undiluted as described previously (Emara et al. 1995) to incubate with the NC blots overnight at room temperature. The mAb used included MF15-4 (serotype O5 B-band specific), MF83-1 (serotype O6 B-band specific), N1F10 (A-band specific), 5c-101 (outer-core specific), and 5c-7-4 (inner-core specific). Secondary antibodies used were goat anti-mouse Fab_2-alkaline phosphatase conjugated and incubated at room temperature for 60 min. The blots were developed with the BCIP/NBT as per the manufacturer's protocols (Sigma). The LPS banding patterns were visualized using the ultrafast silver nitrate-staining method that was described previously (Fomsgaard et al. 1990).

Results

Biofilm characterization

In mono-species biofilms of *P. aeruginosa* ATCC 10145, *P. aeruginosa* PAO1, and *C. albicans* CECT 1472, respectively, no statistical differences could be discerned in each of these cultures between cultures of 24–48 h biofilms in terms of viable cells ($p > 0.05$) and total biomass ($p > 0.05$) (Figure 1A and B). The analysis of the metabolic activity through XTT reduction assays showed that biofilms of both 24 and 48 h *P. aeruginosa* ATCC 10145 exhibited similar activities while biofilms of *P. aeruginosa* PAO1 had lower metabolic activity after 48 h. On the contrary, 48 h old biofilms of *C. albicans* appeared to be metabolically more active than the 24 h old biofilms (Figure 1C).

In comparison with mono-species biofilms of *C. albicans* (Figure 1A), the formation of mixed biofilms showed that *P. aeruginosa* caused a significant inhibition of proliferation of *C. albicans*. This inhibition of *C. albicans* was greater in the presence of *P. aeruginosa* ATCC 10145 which resulted in ~2- and 3-log reduction in the number of viable cells of *C. albicans* in 24 and 48 h old biofilms (Figure 1A), respectively, than in the presence of *P. aeruginosa* PAO 1 strain. However, there was no inhibition of *C. albicans* in a planktonic co-culture with *P. aeruginosa*, since both microorganisms showed similar growth rates when compared to their respective mono-species cultures (data not shown). Neither of the two wild-type strains of *P. aeruginosa* was influenced by the presence of *C. albicans*, since the CFU of the viable cells of *P. aeruginosa* present in dual-species biofilms were similar to the numbers observed in the mono-species biofilms of *P. aeruginosa* (Figure 1A). The total biomass in dual-species biofilms was lower in the 48 h biofilms; this is due to the reduction observed in the number of cells of *C. albicans* (Figure 1B). However, there was no statistical difference in the metabolic activity between 24 and 48 h-old mixed biofilms (Figure 1C).

Examination of the biofilms by SEM showed that there was a clear reduction in the numbers of cells of *C. albicans* in dual-species biofilms compared to mono-species biofilms of *C. albicans* biofilms (Figure 2). Furthermore, on examining the 48 h-old dual-species biofilms formed by *P. aeruginosa* ATCC 10145 and *C. albicans* CECT 1472, it was found that the biofilm of *Pseudomonas* covered the entire surface even masking a few cells of *C. albicans*. Whereas in the biofilms formed by *P. aeruginosa* PAO1 and *C. albicans* CECT 1472, the

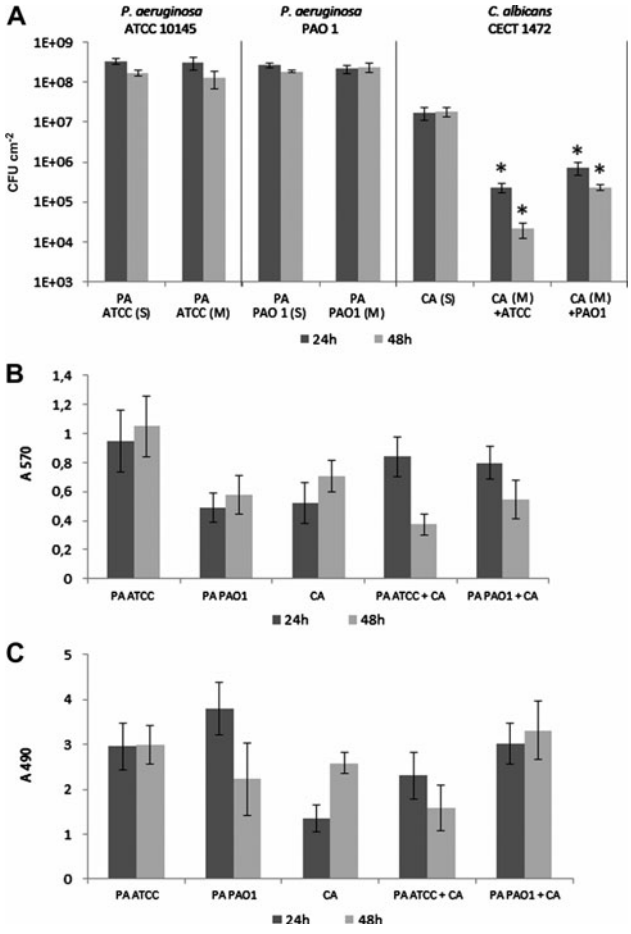

Figure 1. Quantitative assessments of the growth of biofilm of single (S) and mixed (M) cultures. Mono-species biofilms are those of *P. aeruginosa* ATCC 10145 (PA ATCC), PAO1 (PA PAO1), and *C. albicans* CECT 1472 (CA), respectively. Dual-species biofilms are composed of CA with PA ATCC or PA PAO1. Quantitative measurements were made at 24 h (black bar) and 48 h (grey bar). (A) Number of viable cells present in the biofilms; (B) biomass quantification by measuring the intensity of CV stain at A_{570}; (C) evaluation of the metabolic activity of the biofilm by XTT reduction assay by making measurements at A_{490}. Standard deviations are based on two independent experiments, each being performed in triplicate. *Significantly different ($p < 0.05$) from the number of cells in mono-species biofilms.

Candida cells were not covered by *Pseudomonas*. The results obtained clearly showed that *P. aeruginosa* inhibited the formation of biofilms of *C. albicans*.

Infection of biofilms with phage

Both the phages, phiIBB-PAA2 and phiIBB-PAP21, can be considered effective against their target *Pseudomonas* cells in the dual-species biofilms, achieving an ~2- and 1.5-log reduction, respectively, in the number of viable cells of *P. aeruginosa*, 6 h post infection (Figure 3). As expected, the reduction in the mixed biofilms of *P. aeruginosa* caused a reciprocal increase in the amount of cells of *C. albicans*. However, at 24 h post infection of the mixed biofilms, an increase in the number of viable cells of *P. aeruginosa*, ~1.5 log for *P. aeruginosa* ATCC 10145 strain and 1 log for *P. aeruginosa* PAO1, was observed as compared to the CFU numbers 6 h post infection (Figure 3A and B). This suggests that the cells of *P. aeruginosa* acquired resistance to the phages between 6 and 24 h of infection of the biofilm. This increase in *P. aeruginosa* at 24 h of phage infection did not appear to interfere with the growth of the biofilm of *C. albicans* and accordingly a similar increase in the number of *C. albicans* was observed. An increase of 0.5 and 1 log in the CFU of *C. albicans* in the presence of *P. aeruginosa* PAO1 and ATCC 10145, respectively, at the 24 h time point was observed as compared with the initial time point of phage infection (Figure 3A and B). This increase in the number of cells of *C. albicans* after phage infection was also substantiated by the results from SEM analysis (Figure 3E and F). Interestingly, after infecting the dual-species biofilm with phage phiIBB-PAA2, the cells of *C. albicans* changed from the yeast to the filamentous form (Figure 3E). This morphological change, observed at the 24 and 48 h time points, was not observed in mono-species biofilms of *C. albicans* (Figure 2A and 2B).

To understand the reasons behind non-inhibition of the biofilms of *C. albicans* in the presence of cells of *P. aeruginosa* after 24 h of phage infection of the dual-species biofilm, the hypothesis that the lysates of *P. aeruginosa* served to provide nutrients for the proliferation of *C. albicans* was tested. However, no increase in the growth of biofilm of *C. albicans* could be discerned due to the supplementation with the sonicated and phage lysates of *P. aeruginosa* (Figure 4). The tested number of viable cells of *C. albicans* in the biofilms supplemented with both types of lysates of *P. aeruginosa* was 0.5 log lower than that in 48 h old biofilms of *C. albicans*. Hence, these results do not support the hypothesis stated above.

The hypothesis that the resurgence in the growth of cells of *C. albicans* in the phage-infected dual-species biofilms was caused by the emergence of mutant phenotypes of phage-resistant *P. aeruginosa*, which are not able to inhibit the proliferation of the biofilms of *C. albicans*. Four of these strains (M2, M4, M5 and M6) showed resistance to the phage phiIBB-PAA2; and only strain M2 was resistant to phage phiIBB-PAP21 also (Table 2). Dual-species biofilms with *C. albicans* were once again grown with these *P. aeruginosa* ATCC 10145-derived phenotype variants (Figure 5). Furthermore, four defined mutant strains of *P. aeruginosa* PAO1 LPS (wzy, rmd, wbpL and rmlC) were also used to assess the role of LPS in assaying the biofilms of

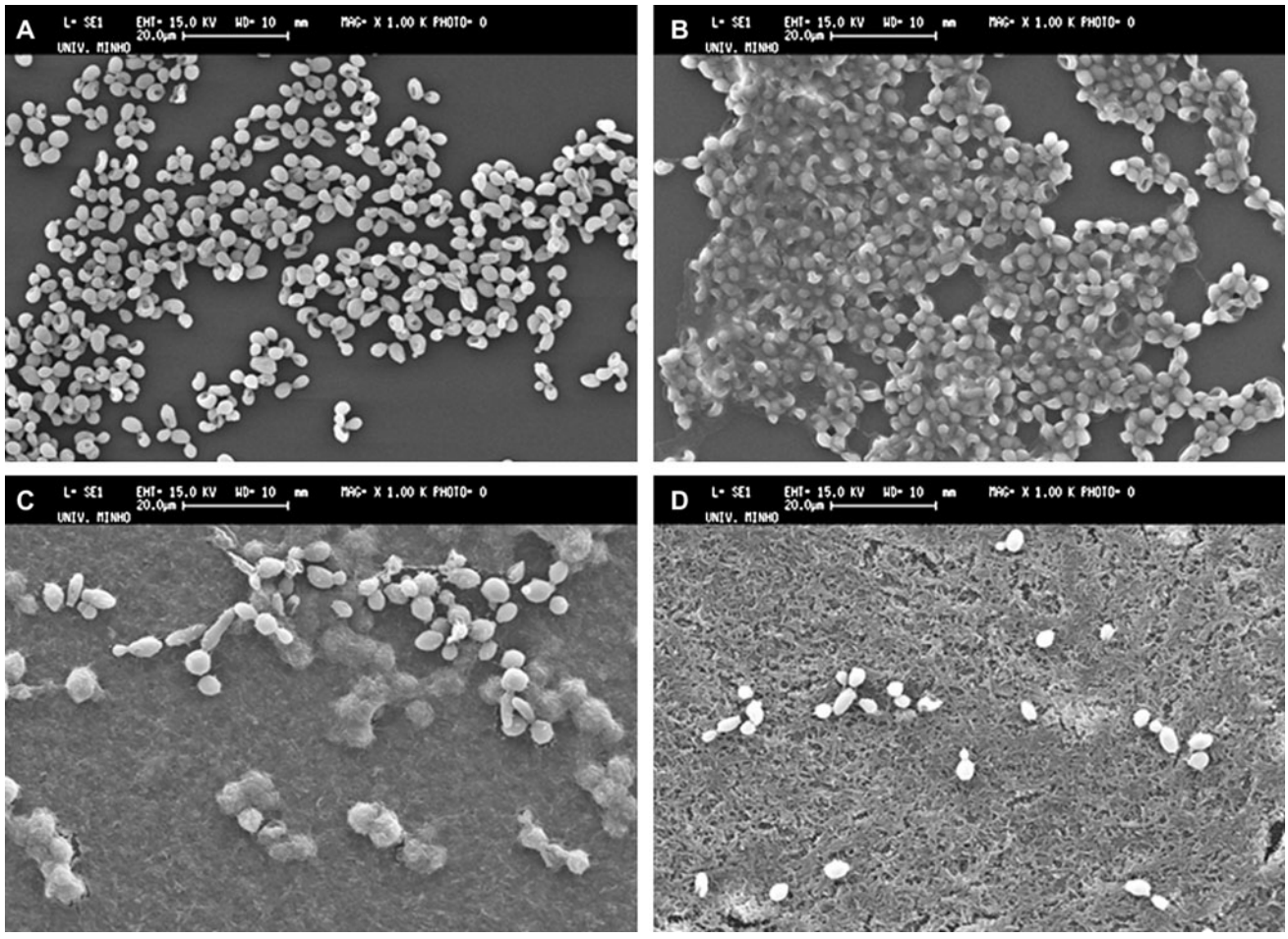

Figure 2. Examination using a SEM of mono- and dual-species biofilms. 24 h (A) and 48 h (B) old biofilms formed by *C. albicans* CECT 1472; (C) 48 h old dual-species biofilms formed by *P. aeruginosa* ATCC 10145 and *C. albicans* CECT 1472; (D) 48 h old dual-species biofilm formed by *P. aeruginosa* PAO1 and *C. albicans* CECT 1472.

C. albicans (Figure 5). These strains were also tested for susceptibility against phages, phiIBB-PAA2 and phiIBB-PAP21, (Table 2) and it was observed that all of them were susceptible to phage phiIBB-PAP21; note that the wild-type strain *P. aeruginosa* PAO1 was also susceptible to this phage. Nevertheless, only the wild-type strain, wzy and rmd mutant strains, were susceptible to phage phiIBB-PAA2. The other two strains (wbpL and rmlC) that have a shorter chain of B-band LPS were resistant to this phage.

Dual-species biofilms formed by the different *P. aeruginosa* ATCC 10145-derived phenotypes variants revealed that most of them could co-inhabit better with *C. albicans* than the parental strain ATCC 10145 (Figure 5). This has led to the observed increases in the growth of *C. albicans* at 24 h post phage infection of the dual-species biofilms. This variation is more pronounced after 48 h of formation of the biofilm (Figure 5B) since the cells in the biofilm of *C. albicans* increased by more than 1 log in the mixed cultures with the M1–M6 strains as compared to co-culture of biofilms of *C. albicans* grown with the parental strain, *P. aeruginosa* ATCC 10145. Nevertheless, the number of cells of *C. albicans* in these biofilms is still ~1 log lower than the numbers obtained in mono-species biofilms of *C. albicans*. A similar result was obtained with dual-species biofilms of mutants of *P. aeruginosa* PAO1 LPS and *C. albicans*. Although the number of cells of *C. albicans* cells after 24 h of formation of the biofilm with these mutants is slightly higher compared to the parental strain *P. aeruginosa* PAO1 (Figure 5A), this increase is more significant after 48 h of formation of mixed biofilm mainly with the mutant rmlC, which has a defect in the biosynthesis of core oligosaccharide and produces the shortest LPS chain among all of the mutants tested (consisting of an inner core and lipid A region) (Figure 5B). The number of cells of *C. albicans* in dual-species biofilm with this LPS-defective strain is

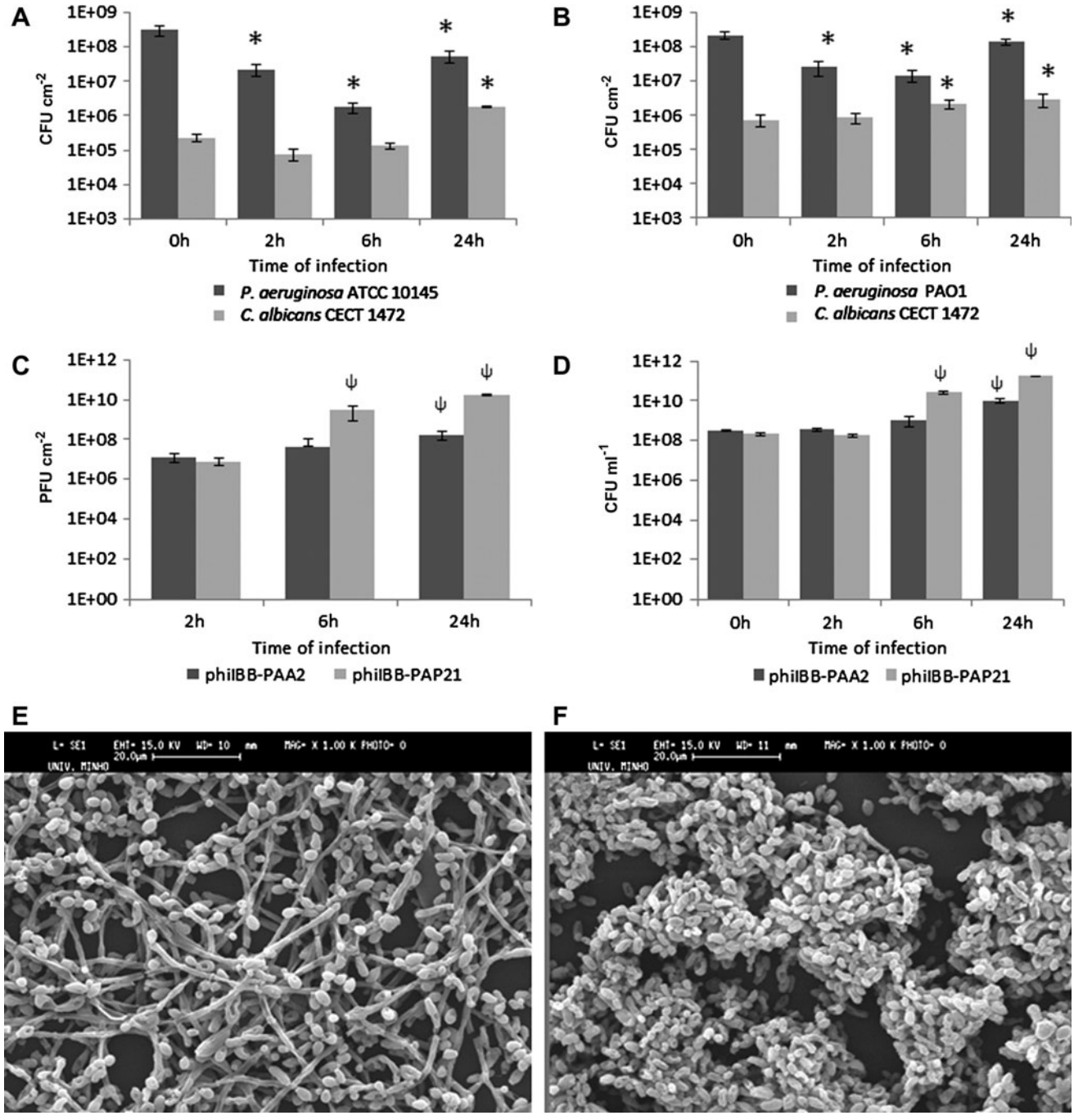

Figure 3. Phage infection of dual-species biofilm with phiIBB-PAA2 and phiIBB-PAP21. CFU counts of the viable cells of *P. aeruginosa* ATCC 10145 and *C. albicans* CECT 1472 (A) or *P. aeruginosa* PAO1 and *C. albicans* CECT 1472 (B) present in mixed biofilms before (0 h) and after infection (2, 6 and 24 h); PFU counts of phages attached to the mixed biofilms after infection (C); and of phages released from biofilms (D); SEM images of: 24 h old biofilms of *P. aeruginosa* ATCC 10145 and *C. albicans* CECT 1472 after 24 h of infection with phage phiIBB-PAA2 (E); and 24 h old biofilms of *P. aeruginosa* PAO1 and *C. albicans* CECT 1472 after 24 h of infection with phage phiIBB-PAP21 (F). Error bars represent standard deviations from two independent experiments performed in triplicate; *significantly different ($p < 0.05$) from the number of cells of the biofilm at 0 h; $^{\Psi}$significantly different ($p < 0.05$) from the number of phages at the beginning of experiment.

only 0.5 log lower compared with 48 h mono-species biofilms of *C. albicans* (Figure 1).

The differences in the phenotype of LPS among the different strains of *P. aeruginosa* including the PAO1

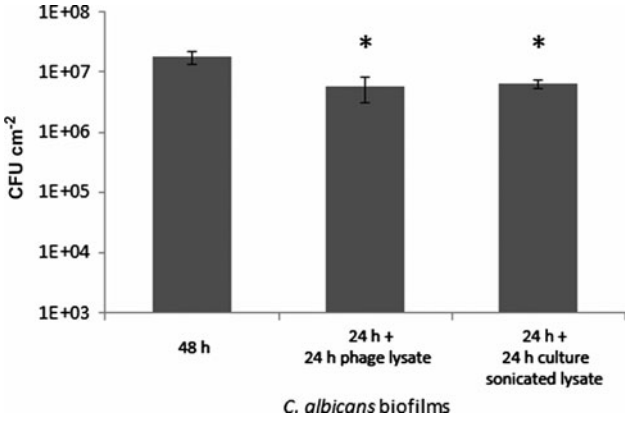

Figure 4. Comparison of 48 h old biofilms of *C. albicans* CECT 1472 with biofilms of *C. albicans* formed over 24 h and exposed for 24 h to *P. aeruginosa* phage or sonicated cultured lysates. Error bars represent standard deviations from two independent experiments performed in triplicate; *significantly different ($p < 0.05$) from 48 h biofilms of old *C. albicans*.

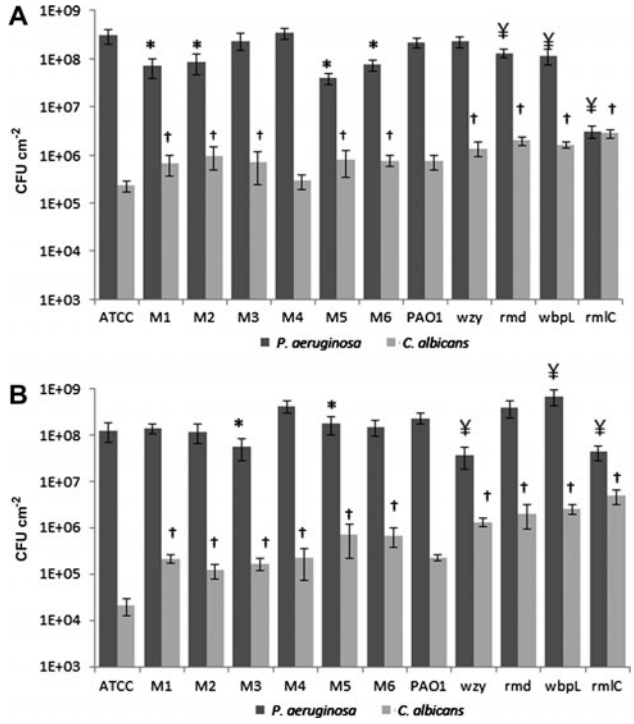

Figure 5. Dual-species biofilms formed by *P. aeruginosa* ATCC 10145 (M1–M6) or PAO1 mutant strains with *C. albicans* CECT 1472. Dual-species biofilms of *C. albicans* CECT 1472 formed with *P. aeruginosa* ATCC and PAO1 phenotypes for 24 h (A) and 48 h (B). Error bars represent standard deviations from two independent experiments performed in triplicate; *significantly different ($p < 0.05$) from the number of cells in the biofilm of wild-type strain of *P. aeruginosa* ATCC 10145; ¥significantly different ($p < 0.05$) from the number of cells in the biofilm of wild-type strain of *P. aeruginosa* PAO1; †significantly different ($p < 0.05$) from the number of cells of biofilm of *C. albicans* CECT 1472 in mixed biofilms with wild-type strains of *P. aeruginosa* (ATCC 10145 or PAO1).

wild type and the defined LPS mutants, wzy, wbpL, and rmlC, are clearly visible (Figure 6). The wzy mutant is devoid of B-band LPS, while the wbpL mutants showed faster migrating bands of the core oligosaccharide reactive with mAb 5c-7-4 (inner-core specific) and no reaction to mAb MF15-4 (B-band specific against serotype O5) or 5c-101 (outer-core specific) indicative of a defect in the outer-core region. Accordingly, the rmlC mutant which causes the lowest inhibition in the cells of *C. albicans* exhibits a LPS banding pattern that is devoid of both A-band and B-band LPS and has a fast migrating band indicative of a truncated LPS core (Figure 6, panel A). The rmd mutant is defective in A-band LPS biosynthesis, hence, as expected did not show any defect in the production of high-molecular-weight (HMW) B-band LPS (Figure 6, panel A). These results verified the defects in the LPS of the mutants as were reported in previous studies by the Lam laboratory. Therefore, the LPS banding patterns from these mutants are useful standards for characterizing the phenotype of the LPS in the M1–M6 mutants. Among the ATCC 10145-derived M1–M6 mutants, the M2 mutant (Figure 6B) is the only strain that is resistant to both phiIBB-PAP21 and phiIBB-PAA2 phages. M2 exhibited a LPS banding pattern that showed a lack of HMW bands, indicating defects in the production of A-band and B-band LPS. In addition, M2 LPS also showed a fast-migrating core oligosaccharide band indicating truncation in the LPS core. Due to truncation of the LPS core, neither A-band nor B-band O-polysaccharides could be assembled onto the core, hence HMW bands of A-band and B-band polysaccharide that are linked to carriers of undecaprenol lipid still remain, as observed in the two immunoblots that were probed with mAb MF83-1 (B-band specific against serotype O6) and mAb N1F10 (A-band specific).

Table 2. Lytic spectra of the 2 phages against the *P. aeruginosa* ATCC and PAO1 mutant phenotypes.

	P. aeruginosa ATCC 10145							*P. aeruginosa* PAO 1				
	Wild type	M1	M2	M3	M4	M5	M6	Wild type	wbpL	rmlC	wzy	rmd
Phage phiIBB-PAA2	+	+	−	+	−	−	−	+	−	−	+	+
Phage phiIBB-PAP21	+	+	−	+	+	+	+	+	+	+	+	+

(+ susceptible; − not susceptible).

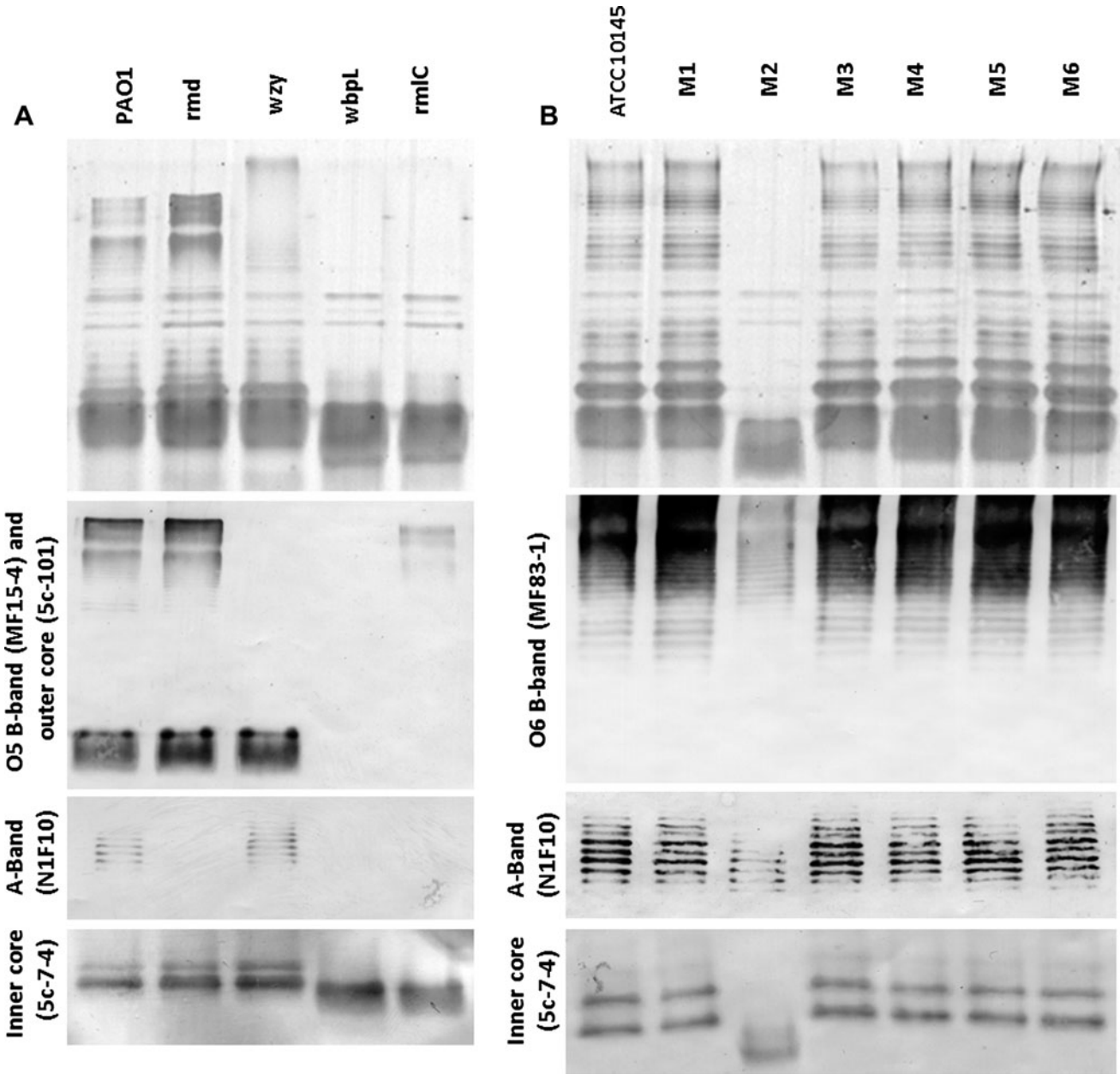

Figure 6. SDS-PAGE and Western immunoblotting analysis of LPS from *P. aeruginosa* PAO1 and PAO1-derived isogenic LPS mutant strains (Panel A) and *P. aeruginosa* ATCC 10145 and M1–M6 phenotype variants (Panel B). Silver stained SDS-PAGE gels were displayed at the top of each of panels A and B, followed by immunoblots that were probed with mAbs, MF15-4 (B-band specific against serotype O5), 5c-101 (outer-core specific), N1F10 (A-band specific), and 5c-7-4 (outer-core specific), and MF83-1 (B-band specific against serotype O6, which is the serotype determined for strain ATCC 10145). Note that in panel A, both mAb MF15-4 and 5c-101 were used simultaneously on one of the blots to demonstrate the truncation in the LPS core in wbpL and rmlC mutants and the lack of B-band LPS in wzy, wbpL, and rmlC mutants. On Panel B, only a B-band specific mAb MF83-1 was used and not mAb 5c-101, as the latter mAb is specific against the terminal glucose of the outer core, which is absent in O6 strains. Note the lack of high-molecular-weight bands and the fast migrating core-oligosaccharide band in the LPS sample from M2.

The nature of these undecaprenol-linked polysaccharide bands has been verified due to their sensitivity to phenol as demonstrated in an earlier study by the authors when LPS was prepared using the standard phenol and hot water method (Rocchetta & Lam 1997). In contrast, the LPS banding patterns from M1 and M3-M6 were all quite similar to the LPS banding pattern of wild-type strain of *P. aeruginosa* ATCC 10145 (Figure 6B) indicating that they have very distinct phenotypes of LPS as compared to M2.

Discussion

C. albicans and *P. aeruginosa* are frequently involved in severe infections associated with polymicrobial biofilms formed on indwelling devices (Pierce 2005). It has been reported that the virulence of *C. albicans* is influenced by the presence of *P. aeruginosa* as this bacterium exhibits antifungal behavior against *Candida* spp. (Bandara, Yau, et al. 2010; Holcombe et al. 2010). However, the interaction between *Candida* spp. and *P. aeruginosa* in a polymicrobial biofilm is not fully understood. In the present work, inhibition in the development and filamentation of *C. albicans* was observed in mixed biofilms with *P. aeruginosa*. After 24 and 48 h of formation of the mixed biofilm (Figure 1), the amount of viable cells of *C. albicans* was significantly reduced compared with single biofilms of *C. albicans*. However, the substantial reduction in the count of *C. albicans* reported by Bandara et al. after 48 h of incubation of mixed biofilm with *P. aeruginosa* was not observed in the authors' study (Bandara, Yau, et al. 2010). Interestingly, no significant changes occurred in the number of viable cells of *P. aeruginosa* between 24 and 48 h dual-species and mono-species biofilms of *P. aeruginosa*. Similar results were observed by other authors who showed that *P. aeruginosa* forms a dense biofilm on the filaments of *C. albicans*; presumably this superimposed biofilm of *P. aeruginosa* is responsible for the destruction of the fungus (Hogan & Kolter 2002).

In recent years, many studies have shown the potential of using phages as antibacterial agents for the treatment of infectious diseases (Weber-Dabrowska et al. 2001, 2003; Matsuzaki et al. 2005; Hanlon 2007; McVay et al. 2007) and for control of bacterial biofilm (Tait et al. 2002; Curtin & Donlan 2006; Sillankorva et al. 2008; Donlan 2009). The phages used in this study were previously isolated from hospital effluents and were shown to be capable of infecting a variety of strains of *P. aeruginosa* that are resistant to antibiotics and also to control both exponential- and stationary-phase cells (Pires et al. 2011). The infection of dual-species biofilms with phage resulted in a significant reduction of viable cells of both *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO1 as early as 2–6 h post infection. However, after the initial reduction in the count of bacterial cells, the number of viable cells of *P. aeruginosa* was found to increase at the 24 h time point (Figure 3). This could be interpreted as the cells of *P. aeruginosa* acquiring resistance to the respective phages. On the other hand, the number of cells of *C. albicans* present in the dual-species biofilm steadily increased from the time of phage infection and achieved significantly higher numbers of viable cells after infection of the biofilm for 24 h. These results suggest that the surviving cells of *P. aeruginosa* after the phage attack have acquired changes in their phenotype and exhibited a diminished ability to inhibit the growth of biofilm of *C. albicans*. Another possible explanation is that, after phage infection, the reduction in the number of cells of *P. aeruginosa* present in the biofilm increased the source of nutrients available for the proliferation of *C. albicans*. However, after adding the lysates of *P. aeruginosa* to biofilms of *C. albicans* (Figure 4), it was observed that the results did not support this hypothesis. Nevertheless, the results showed a slight decrease in the biofilms of *C. albicans*, suggesting that lysates containing intracellular soluble material from *P. aeruginosa*, like LPS, inhibit the proliferation of biofilm.

The phenotype variants of *P. aeruginosa* ATCC 10145, M1–M6, isolated during the course of this work, the majority of which (4 out of 6) were phage-resistant (Table 2), were found to exhibit a significantly reduced ability to inhibit the proliferation of biofilm of *C. albicans* as compared to the co-culture experiments using the wild-type strain of the parental *P. aeruginosa* ATCC 10145. Since several studies have shown that LPS molecules are the receptors of T7-like phages and changes in the LPS structure is a major cause for bacterial resistance to phages (Wright et al. 1980), one of the explanations for the reduced influence of the phage-resistant phenotypes *P. aeruginosa* on the growth of *C. albicans* is a possible modification of the LPS, an integral component of the cell envelopes and a major factor for virulence (Lam et al. 2011). Thus, the LPS of these ATCC-10145-derived phenotype variants were extracted and analyzed together with a number of defined LPS knockout mutant strains, provided by one of the co-authors, from the collection of strains of the University of Guelph. Among the ATCC 10145-derived phentoype variants of *P. aeruginosa* that were isolated post phage infection, most of these strains exhibit an LPS banding profile in SDS-PAGE and Western immunoblotting analysis (Figure 6) that was quite similar to the pattern observed in the parental ATCC strain. Only the LPS banding pattern of the *P. aeruginosa* ATCC_M2 strain, which was resistant to both the phages tested, revealed a significant difference in the reduction of the visible A-band or B-band LPS and a faster migrating band of the core oligosaccharide. It has been reported that bacterial LPS have a significant effect on the formation of biofilm by *Candida* species (Bandara et al. 2009; Bandara, Lam, et al. 2010). According to Bandara et al. (2013) bacterial LPS alter the gene expression of *C. albicans* during the development of the biofilm, influencing directly the proliferation of the biofilm and formation of hyphae. The observation revealed that the mutant rmlC, which was truncated at the core oligosaccharide and therefore lacking the outer core region and the chains of both A-band or B-band O polysaccharide, was the one exhibiting the lowest inhibition in the proliferation of *C. albicans*. Therefore, these observations are in good

agreement with observations that were made in the previous study (Bandara, Lam, et al. 2010). Nevertheless, as shown in this work the phage-resistant phenotypes with the exception of M2 did not exhibit any modification in their LPS and yet they did not inhibit the formation of biofilms of *C. albicans* compared to the wild-type strains. It was therefore hypothesized that the phage-resistant phenotypes have alterations in their surface receptors as pili, flagella or surface proteins which have been previously described as phage receptors (Kropinski 2008; Ceyssens et al. 2011; Kim et al. 2012). These receptors may also be the cause of the inhibition of proliferation of biofilm of *C. albicans*. Further experiments are needed to confirm this hypothesis.

In conclusion, the work described here showed that *P. aeruginosa* effectively inhibits the proliferation of biofilms of *C. albicans* due to the presence of bacterial LPS that exert suppressive effects towards the cells of *C. albicans*. Consequently, *C. albicans* is able to co-inhabit better with mutant strains of *P. aeruginosa*, which only contain the truncated core and lipid-A region of the LPS chain. Hence, the data showed a correlation between the inhibition of the formation of biofilm of *C. albicans* and the composition of the LPS chain of *P. aeruginosa*.

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