Metal resistance in *Candida* biofilms

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Abstract

Yeasts are often successful in metal-polluted environments; therefore, the ability of biofilm and planktonic cell *Candida tropicalis* to endure metal toxicity was investigated. Fifteen water-soluble metal ions, chosen to represent groups 6A to 6B of the periodic table, were tested against this organism. With in vitro exposures as long as 24 h, biofilms were up to 65 times more tolerant to killing by metals than corresponding planktonic cultures. Of the most toxic heavy metals tested, only very high concentrations of Hg²⁺, CrO₄²⁻ or Cu²⁺ killed surface-adherent *Candida*. Metal-chelator precipitates could be formed in biofilms following exposure to the heavy metals Cu²⁺ and Ni²⁺. This suggests that *Candida* biofilms may adsorb metal cations from their surroundings and that sequestration in the extracellular matrix may contribute to resistance. We concluded that biofilm formation may be a strategy for metal resistance and/or tolerance in yeasts.

Keywords

*Candida tropicalis*; biofilm; metal tolerance; antifungal resistance; metalloid; extracellular polymeric matrix.

Introduction

In ecological studies of yeast populations, *Candida* represents an abundant fungal genus found in rich soil and aquatic habitats (Slavikova & Vadkertiova, 1997; Ahearn, 1998; Lopez-Archilla et al., 2004). Toxic metals may be naturally spread into these places by volcanic activity or by the weathering of minerals (Brown et al., 2003). Urbanization and industrialization have also disseminated water-soluble metal ions into our environment, including into forests, rivers and even protected regions that may otherwise be considered pristine (De Vries et al., 2002; Hernandez et al., 2003; Schparyk & Parpan, 2004). As examples, Zn²⁺, Ni²⁺ and Mn²⁺ have been reported at concentrations of 19, 6.5 and 39 mM, respectively, in the mine-polluted Huelva estuary, Spain (Morillo et al., 2005). In the severely contaminated River Avoca in southeast Ireland, the heavy metals Cu²⁺ and Pb²⁺ are present in the subsurface sediment at 14.2 and 2.2 mmol g⁻¹, respectively (Gaynor & Gray, 2004). Yeasts often become the predominant microbial species in aqueous locales contaminated by heavy metal pollutants (Hagler & Mendocs-Hageler, 1981).

*Candida* spp. have been isolated from metal-contaminated pulp-mill wastewater and acid mine runoff (Suikho & Hoekstra, 1999; Lopez-Archilla et al., 2004). *Candida albicans* and *Candida tropicalis* are known for high levels of resistance to the water-soluble ions Hg²⁺, Pb²⁺, Cd²⁺, arsenate (AsO₄³⁻) and selenite (SeO₃²⁻) (Berdicevsky et al., 1993). Many *Candida* spp. also have the capacity to adsorb and/or accumulate metals from their surroundings (Podgorskii et al., 2004). Metaloadsorption by nonpathogenic and cell-surface engineered microbes is a promising technology for the bioremediation of metal-polluted sites (Costley & Wallis, 2001; Lee et al., 2003). Our work is focused on the ability of *C. tropicalis* biofilms to survive exposure to metals and adsorb them from an aqueous growth medium.

The majority of microorganisms found in medical, industrial and natural environments grow in multicellular, surface-adherent assemblages termed biofilms. Approximately 10% of *Candida* spp., including *C. tropicalis*, are opportunistic human pathogens that are normal inhabitants of human skin, and vaginal, gastrointestinal and urinary tracts (Ahearn, 1998). The majority of studies of *Candida* biofilms have been centred on medical applications. Candidiasis is most often associated with biofilm formation on indwelling medical devices (Nyguyen et al., 1995; Chandra et al., 2001a; Kojic & Darouiche, 2004). *Candida* spp. are frequently recovered from hard surfaces in hospitals as well as from sites of fecal contamination (Eggimann et al., 2003). Within these settings, *Candida* biofilms are infamous for...
high levels of resistance to disinfectants and antifungal agents (Lamfon et al., 2004; Ramage et al., 2005).

Although the tolerance of bacterial biofilms to metal ions has been documented in a number of studies (Teitell & Parsek, 2003; Harrison et al., 2004a, b, 2005b, c), no study to date has specifically examined the susceptibility of yeast biofilms to metals. This study employed a high-throughput method previously developed by our research group to examine the susceptibility of bacterial biofilms to antibiotics, disinfectants and metals (Ceri et al., 1999; Harrison et al., 2004a). Here we modified this technique to examine the ability of C. tropicalis biofilms to survive exposure to a diverse array of toxic metal ions. Candida tropicalis biofilms survived (and in some instances grew) in high concentrations of these antimicrobials, whereas corresponding planktonic cells died in a time-dependent fashion. Furthermore, the ability of yeast biofilms to adsorb metals was investigated. When treating exposed biofilms with the organic chelator diethyldithiocarbamate, highly coloured metal precipitates rapidly became visible in these surface-adherent microbial communities. We suggest that biofilm formation may function as a mechanism of metal resistance and/or tolerance for Candida.

Materials and methods

Strains and growth media

Candida tropicalis 99916 (an isolate from the Foothills Hospital in Calgary, AB, Canada) was stored at −70°C in a MicrobankTM (ProLab Diagnostics, Toronto, Canada) according to the manufacturer’s directions. This yeast was grown in tryptic soy broth (TSB, EMD Chemicals Inc., Gibbstown, NJ) for all metal susceptibility assays. Subcultures, fungicidal assays and viable cell counts were carried out by plating on tryptic soy agar (TSA) (EMD Chemicals Inc.), and incubating at 30°C for 48 h. Serial dilutions were performed using 0.9% saline.

Biofilm cultivation

Biofilms were grown in the MBEC™ Physiology and Genetics assay (MBEC BioProducts Inc., Edmonton, Canada; http://www.mbec.ca). This method was previously described by Ceri et al. (1999, 2001), and uses a plastic lid with 96 pegs that fits inside a standard 96-well microplate. This device and the cultivation methods were modified to facilitate the growth of yeast biofilms. The peg lid was immersed into a sterile solution of 1% L-lysine in double-distilled water (ddH2O) and incubated at room temperature for 16 h. The lids were then dried upside down in a laminar-flow hood for 30 min. Coating the pegs with a positively charged amino acid enhanced the ability of C. tropicalis 99916 to form biofilms on this polystyrene surface.

Starting from cryogenic stocks, C. tropicalis was streaked out twice on TSA. An inoculum was prepared by suspending colonies from the second agar subculture in TSB to match a 1.0 McFarland standard. This standard inoculum was diluted 30-fold in TSB to attain a starting viable cell count of roughly 1.0 × 10^6 CFU mL⁻¹. One hundred and fifty microliters of this inoculum was transferred into each well of a 96-well microtitre plate. The dried, L-lysine-coated peg lids were then inserted into the microplates containing this inoculum. These devices were placed on a gyrorotary shaker at 125 rpm for 48 h in an incubator at 35°C and 95% relative humidity.

Biofilms were rinsed once with 0.9% saline (by placing the lid in a microplate containing 200 µL of saline in each well) to remove loosely adherent planktonic cells. Biofilm formation was evaluated by breaking four pegs from each device after it had been rinsed. Biofilms were disrupted into 200 µL of 0.9% saline using a water table sonicator on the setting ‘high’ for a period of 5 min (Aquasonic model 250 HT; VWR Scientific, Mississauga, Canada). Alternatively, microbial growth was evaluated for statistical equivalence by disrupting all biofilms at the same time from the lid into a microplate with 200 µL of saline in each well. The disrupted biofilms were serially diluted and plated onto agar for viable cell counting.

Stock solutions of metal compounds

Sodium arsenite (NaAsO₂), cadmium sulphate (CdSO₄·8/3 H₂O), lead nitrate [Pb(NO₃)₂], manganese chloride (MnCl₂·6H₂O), nickel sulphate (NiSO₄·6H₂O), potassium selenite (Na₂SeO₃), silver nitrate (AgNO₃), and potassium tellurite (K₂TeO₄) were obtained from Sigma Chemical Company (St Louis, MO). Aluminum sulphate [Al₂(SO₄)₃·18 H₂O], sodium hydrogen arsenate (Na₃H₂AsO₄), cupric sulphate (CuSO₄·5H₂O), potassium dichromate (K₂Cr₂O₇), mercuric chloride (HgCl₂), and zinc sulphate (ZnSO₄·7H₂O) were obtained from Fischer Scientific (Ottawa, Canada). Cobalt chloride was purchased from British Drug Houses Limited (Poole, England). All metal compounds were dissolved in ddH₂O to obtain a stock solution of the metal counterion at a concentration five times the maximum used in susceptibility assays. These solutions were passed through 0.22 µm syringe filters into sterile glass vials and stored at room temperature until used. Working solutions of metals were prepared in TSB from stock metal cation or oxyanion solutions not more than 60 min prior to exposure.

Metal susceptibility testing of biofilms

The rinsed peg lids were transferred to 96-well microtitre plates containing serial twofold dilutions of metal compounds in TSB. The first and last well of every row served as a sterility and growth control, respectively. These plates were incubated at 35°C and 95% relative humidity for 5 or
24 h. Biofilm metal susceptibility testing was performed according to the method of Harrison et al. (2004a). All metal susceptibility assays used a neutralizing step as previously described (Harrison et al., 2004a, b, 2005b). Co^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and all metal oxyanions were neutralized using 10 mM reduced glutathione (Harrison et al., 2004a, b), Cu^{2+} and Ni^{2+} were chelated with 0.5 mM sodium diethyldithiocarbamate (DDTC) (Harrison et al., 2004a, 2005c), a maximum concentration dictated by its toxicity to yeast cells. Minimum lethal concentrations for planktonic cells (MLCp) and biofilms (MLCb) were redundantly determined without the use of this agent. Silver was complexed with 10 mM sodium citrate, Al^{3+} and Mn^{2+} with ~1–2 mM acetylsalicylic acid, and Hg^{2+} with 10 mM l-cysteine (Harrison et al., 2004a). All neutralizing agents were purchased from Sigma Chemical Company.

Following exposure, peg lids were rinsed twice with 0.9% saline, and the biofilms were disrupted into fresh recovery media (which contained the neutralizing agents). Twenty-microlitre aliquots of the recovery cultures (containing the disrupted biofilms) were plated onto TSA. These plates were incubated for 48 h and were scored for growth to obtain MLCB values. Alternatively, log-killing was evaluated by serial dilution of the recovery cultures and by plating onto TSA.

**Planktonic cell susceptibility testing**

To evaluate planktonic minimal inhibitory concentration (MIC) and MLCp values we used a modified protocol for yeast susceptibility testing suggested by the American Society for Microbiology (Fothergill & McGough, 1995). Challenge plates of metals were prepared as described above. A 1.0 McFarland standard was prepared in a manner identical to that used for biofilm cultivation. This standard was diluted twofold in TSB, and 5 μL of this was added to each well of the challenge plates. Forty-microlitre aliquots of the planktonic cultures were transferred to 96-well microtitre plates (which contained neutralizing agents). Twenty-microlitre aliquots of the neutralized planktonic cultures were plated onto TSA. These plates were incubated for 48 h and scored for growth to obtain MLCp values. Alternatively, log-killing was evaluated by serial dilution of neutralized cultures and plating onto TSA. MIC values were obtained after 48 h incubation at 35°C by reading the optical density at 650 nm (OD_{650}) of the challenge plates using a THERMODmax microplate reader with SOFTmax Pro data analysis software (Molecular Devices Corporation, Sunnyvale, CA).

**Confocal laser scanning microscopy (CLSM)**

Pegs were broken from the lid of the MBEC™ assay. A portion of these pegs were immersed in 200 μg mL⁻¹ tetra-methylrhodamine conjugated concanavalin A (TRITC) (Con A, Molecular Probes, Burlington, Canada) and incubated at 30°C for 90 min. Con A is a lectin with high specificity for mannose sugars present in the cell walls and biofilm matrix of *Candida albicans* (Jin et al., 2005). These pegs were subsequently treated with the nucleic acid stain Syto 9 (Molecular Probes) at the dilution of the commercially available stock suggested by the manufacturer. Biofilms were incubated in Syto 9 at 30°C for 20 min. The remaining pegs were stained by immersion into 200 μL of 0.05% weight in volume (w/v) acidine orange (Sigma Chemical Co.) for 5 min at room temperature. Acidine orange is a nucleic acid stain that intercalates dsDNA and binds ssDNA through dye-base stacking (Bernas et al., 2005).

Cell viability staining of yeast using the Live/Dead BacLight™ Kit (Molecular Probes) was carried out according to the method of Jin et al. (2005). Biofilms exposed to metals were rinsed twice with 0.9% saline. Subsequently, biofilm pegs were stained concomitantly with Syto 9 and propidium iodide at 30°C for 30 min. Propidium iodide (green emission) stains all cells in the microbial population regardless of viability. This technique relies on the impermeability of the yeast membrane to propidium iodide (PI, red emission), which only penetrates and stains cells with compromised membrane integrity. Thus live cells stain green, and dead cells stain orange-red. This has been previously shown to correlate well with viable cell counts of a calibrated suspension of *C. albicans* cells (Jin et al., 2005).

Fluorescently labelled biofilm pegs were placed in 2 drops of phosphate-buffered saline (pH 7.2) on the top of a glass coverslip. For Live/Dead staining, 0.9% saline was used instead. These pegs were examined using a Leica DM IRE2 spectral confocal and multiphoton microscope with a Leica TCS SP2 acoustic optical beam splitter (AOBS) (Leica Microsystems, Richmond Hill, Canada). To eliminate artifacts associated with single-wavelength excitation, samples were sequentially scanned, frame by frame, first at 488 nm and then at 543 nm. Fluorescence emission was then sequentially collected in the green and red regions of the spectrum, respectively, for the dye pairs Syto 9+PI, and Syto 9+TRITC-Con A. A 63× water immersion objective was used in all imaging experiments. Image capture and three-dimensional reconstruction of z-stacks were performed using Leica Confocal Software Lite (LCS Lite, available free of charge from Leica Microsystems at http://www.leica-microsystems.com/website/lms.nsf).

**Precipitation of heavy metal cations in *Candida* biofilms**

The organic chelator sodium diethyldithiocarbamate (DDTC) causes the rapid precipitation of some transition metals as well as tellurite in vitro. In particular, DDTC causes...
Cu$^{2+}$, Ni$^{2+}$ and TeO$_3^{2-}$ to form brown, light-yellow, and dark-yellow precipitates, respectively (Cheng et al., 1982; Turner et al., 1992). Our research group has previously exploited this to show that metal cations may be retained inside bacterial biofilms (Harrison et al., 2005b). To test if this also occurred for biofilms of yeast, Candida tropicalis was grown on the pegs of the MBECTM device and exposed to Cu$^{2+}$, Ni$^{2+}$ or TeO$_3^{2-}$ for 5 h. Next, the exposed biofilms were rinsed twice with 0.9% saline for 10 min each time. The peg lids were then inserted into a 96-well microtitre plate with 200 µL of 37.5 mM DDTC in each well. Planktonic cultures in metal challenge media were also exposed to DDTC as a positive control. In this case, 12.5 µL aliquots from wells of the challenge plate (following biofilm exposure) were mixed with 37.5 µL of 150 mM DDTC in the wells of a microtitre plate.

**Statistical tests and data analysis**

One-way analysis of variances (ANOVA) was used to analyse log$_{10}$-transformed raw data with MINITAB® Release 14 (Minitab Inc., State College, PA). Mean and standard deviation calculations of MIC, MLC$_P$, and MLC$_B$ values were performed using Microsoft® Excel XP (Microsoft Corporation, Redmond, WA).

**Results**

**Definitions of measurements**

The minimum lethal concentration (MLC) has been defined as the concentration of an antimicrobial required to kill 99.95% of the fungal population (Fothergill & McGough, 1995). This definition will be retained for planktonic cell susceptibility assays and will be referred to here as MLC$_P$. The minimum lethal concentration for biofilms (MLC$_B$) will be similarly defined here as the concentration of an antimicrobial required to kill 99.95% of the fungal biofilm population. In terms of our assays, this implies that any plate with 1 or no colonies (from a 20 µL test spot) was scored negative for growth. Here we will define the fold tolerance as the ratio of MLC$_B$:MLC$_P$. This measurement is quantitative and also represents a logical argument. If the MLC$_B$ is greater than the maximum concentration examined and the MLC$_P$ is known, then the fold tolerance is greater than or equal to twice the ratio of the highest concentration examined over the MLC$_P$. This takes into account the sensitivity of the assays on a log$_{10}$ scale. Strictly speaking, resistance is defined as the ability of a microorganism to continue growing in the presence of an antimicrobial compound. Tolerance is defined as the ability of a microorganism to survive exposure to, but not grow in the presence of, an antimicrobial.

**Biofilm formation and inocula for susceptibility assays**

For each MBECTM assay, four pegs were broken from the peg lid and the biofilm growth was evaluated by viable cell counting (this gave a total of 168 control measurements). Candida tropicalis grew to a mean cell density (with standard deviation) of 4.3 ± 0.5 log$_{10}$ CFU peg$^{-1}$. To ensure that biofilm growth was even across the rows of pegs in the MBECTM device, all of the biofilms from a single peg lid were plated for viable cell counting. Growth of C. tropicalis biofilms was statistically equivalent between the different rows of pegs by means of one-way ANOVA ($P = 0.402$). These data are presented in Fig. S1 (available as supplementary material). For planktonic susceptibility assays, each well of a separate set of challenge plates was inoculated with 4.5 ± 0.1 log$_{10}$ CFU of planktonic C. tropicalis (a starting number derived from six viable-cell-counting assays of the inoculum). This starting cell number was calibrated to imitate the initial biofilm cell density.

Candida tropicalis biofilms were examined in situ using confocal laser-scanning microscopy (CLSM). Three different, complementary staining techniques were used. In one set of experiments, acridine orange was used to stain C. tropicalis on the pegs of the MBECTM device (green, Figs 1a and d). Biofilms were approximately 7–30 µm in thickness, forming layers and ridges of cells that covered the peg heterogeneously. In a separate set of experiments, Syto 9 (green, Fig. 1b) and TRITC–Con A (red, Fig. 1c) were concomitantly used to label biofilms. Con A is a glucose- and mannose-specific lectin that has been used to label the extracellular polymeric matrix and cell walls of Candida (Chandra et al., 2001a; Jin et al., 2005). Correlative with previous reports, we see that Con A highlighted cell walls and stained EPS to a lesser extent (Chandra et al., 2001a). An overlay of these pictures shows cells in physical contact with their neighbours, joined either by their cell walls or a thin layer of EPS (Fig. 1e). Although the majority of the fungal biomass was composed of yeast cells, some sparsely distributed pseudohyphae and hyphal structures could be observed near the air–liquid-surface interface of the pegs in some instances.

**Susceptibility of Candida tropicalis biofilms to metals**

Means and standard deviations (SD) for metal cation and oxyanion MIC, MLC$_P$ and MLC$_B$ values observed for Candida tropicalis planktonic and biofilm cultures are summarized in Table 1. Frequently, the same value was obtained from every trial for the same compound (i.e. SD = 0). Values reported represent four to eight independent trials each.

Planktonic C. tropicalis was not killed by exposure to three of the metals tested: Ni$^{2+}$, Zn$^{2+}$ and Al$^{3+}$ (corresponding to maximum concentrations of 280, 490 and...
cases of CrO$_4^{2-}$, Mn$^{2+}$, Co$^{2+}$, Cd$^{2+}$, AsO$_4^{3-}$ and AsO$_2^-$, there was a minimum fold decrease in the MLC$_P$ of 8.3, 4.0, 33, 7.7, 4.1 and 2.6, respectively (between 5 and 24 h exposure). These data indicate that planktonic 	extit{C. tropicalis} exhibits time-dependent tolerance to high concentrations of metals. Mercury (Hg$^{2+}$) was the most toxic heavy metal tested, inhibiting yeast growth and killing both planktonic cells and biofilms over a narrow concentration range of 0.5–2.0 mM. As the sole exception, biofilms were no more tolerant to Hg$^{2+}$ than planktonic cells.

Biofilm 	extit{C. tropicalis} was highly tolerant to metal toxicity, surviving in vitro concentrations of more than 120, 140 and 500 mM of the heavy metal(loid)s AsO$_4^{3-}$, Co$^{2+}$ and SeO$_3^{2-}$, respectively. As another example, cationic silver (Ag$^+$) was highly toxic to planktonic cells, inhibiting growth and killing this form of 	extit{Candida} at concentrations of approximately 0.5–1.0 mM and 20–25 mM, respectively. In contrast, biofilms were highly tolerant to Ag$^+$ and were not killed at the highest concentration added in vitro (150 mM). Given the sensitivity of this assay on a log$_2$ scale, this implied that 	extit{C. tropicalis} biofilms were minimally 17–26 times more tolerant to cationic silver than corresponding planktonic cells. Notably (in comparison with planktonic cells), MLC$_B$ values did not decrease with increasing exposure time to metals. Because there was no time-dependent eradication of these surface-adherent microbes, logically, biofilms may have been resistant and/or tolerant to these compounds.

**Killing of 	extit{Candida tropicalis} by Co$^{2+}$ and SeO$_3^{2-}$**

To differentiate between tolerance and resistance in fungal biofilms, the lethality of one metal cation and one metalloid oxyanion was evaluated using viable cell counting and Live/Dead staining in conjunction with CLSM. Co$^{2+}$ was selected as a representative heavy metal cation, because 	extit{Candida} biofilms had the greatest fold tolerance ($\geq 65$) to this compound. SeO$_3^{2-}$ was chosen as a representative oxyanion, because relative to Co$^{2+}$ (as well as to other metals) planktonic cells were not killed time-dependently. Biofilms were at least 21 times more tolerant to SeO$_3^{2-}$ than planktonic cells at both 5 and 24 h exposure (Table 1).

Mean viable cell counts and log-killing of 	extit{C. tropicalis} biofilm and planktonic cells by Co$^{2+}$ are presented in Fig. 2. At concentrations above the planktonic MIC (4.3 mM), both planktonic and biofilm cells were killed by Co$^{2+}$. In the case of planktonic cells, every cell in the population had died by 24 h exposure to 8.6 mM of this heavy metal. In contrast, although a portion of the biofilm population died on exposure to high concentrations (35 or 140 mM) of Co$^{2+}$, there were many survivors even after an in vitro exposure of 1 day (Fig. 2d–h). The size of yeast cells exposed to Co$^{2+}$ for 24 h was larger than that in growth controls (compare Fig. 2c or f–h). There was some differentiation of
Periodic was reflected in a marked decrease in mean viable cell counts and log-killing of Candida tropicalis was rapidly killed (which correlated closely with the corresponding increase in the number of live cells in the biofilm). CLSM of Live/Dead stained biofilms exposed to SeO$_3^{2-}$ (Fig. 3d) showed a corresponding increase in the number of live cells in the biofilms, which generally gave greater surface coverage of the peg. By 24 h exposure to 125 mM SeO$_3^{2-}$ (Fig. 3g), mean viable cell counts of biofilms were similar to the starting number of cells in biofilms. In contrast, 500 mM SeO$_3^{2-}$ generated greater surface coverage of the peg, and the biofilm covered a smaller surface area than before exposure. These data indicate that, under certain conditions, biofilm C. tropicalis was more resistant to SeO$_3^{2-}$ than planktonic cells.

**Retention of divalent heavy metal cations in yeast biofilms**

The ability of bacterial biofilms to trap metal cations has been previously reported (Harrison et al., 2005b). We thus
Fig. 2. *Candida tropicalis* 99916 biofilms are highly tolerant to the heavy metal Co$^{2+}$. Biofilms were grown for 48 h on the peg lid of the MBEC™-high throughput device before exposure for 5 or 24 h to Co$^{2+}$. In a separate series of assays, an equivalent number of planktonic cells were added to metal challenge media. Biofilm and planktonic cells were recovered in saline, serially diluted 10-fold, and then plated onto agar for viable cell counts. Each data point is the mean of three or four independent replicates, and error bars denote standard deviation. An asterisk denotes complete killing of the fungal culture at the reported concentration. Confocal laser scanning microscopy (CLSM) and Live/Dead staining were used to complement cell-counting assays of biofilms on the pegs. (a) Mean viable cell counts of *C. tropicalis* with respect to concentration of Co$^{2+}$. (b) Log-killing of biofilm and planktonic cells. (c) Biofilm before exposure to Co$^{2+}$. The vast majority of biomass was yeast cells and the vast majority were alive. (d and e) Biofilm after 5 h exposure to 35 and 140 mM Co$^{2+}$, respectively. The number of cells that were dead increased with the concentration of heavy metal cations in the growth medium. (f) Biofilm after an additional 24 h incubation, but not exposed to metals. There was some differentiation of yeast into pseudohyphae and hyphae. (g and h) Biofilm after 24 h exposure to 35 and 140 mM Co$^{2+}$, respectively. There were fewer cells and less biofilm coverage of pegs in samples exposed to Co$^{2+}$ for 24 h. Yeast cells were notably larger at 24 h exposure to Co$^{2+}$ than at 5 h. Planktonic cell populations were killed (completely) in a time-dependent fashion by Co$^{2+}$. Many biofilm cells were killed by prolonged exposure to metals. However, some cells in the biofilm population survived exposure to this highly toxic metal even with 1 day of exposure in vitro. Each CLSM panel represents a 2-dimensional average of a 3-dimensional z-stack with a length and width of 238.1 μm taken at a magnification of ×630.
investigated whether yeast biofilms may similarly sequester metal cations or oxyanions from their aqueous surroundings. Biofilms exposed to the metals Cu$^{2+}$, Ni$^{2+}$, and TeO$_3^{2-}$/C0 were rinsed and then treated with the chelator diethyldithiocarbamate (DDTC). This is pictured in Fig. 4. In Cu$^{2+}$ and Ni$^{2+}$ exposed biofilms, DDTC caused the rapid formation of brown and yellow metal-chelates, respectively. However, biofilms exposed to the metalloid oxyanion tellurite did not
change colour when immersed in DDTC. This suggests that *C. tropicalis* biofilms may adsorb positively charged metal ions. Conversely, negatively charged metal ions may not be sequestered in the extracellular matrix of *Candida* biofilms.

**Discussion**

Species of the dimorphic yeast *Candida* are prevalent in soil and aquatic environments. In these niches, microorganisms are frequently exposed to highly toxic, water-soluble metal ions. This study focused on a high-throughput *in vitro* system for examining the susceptibility of yeast biofilms to metals. Two complementary but different approaches were used to examine and compare planktonic cells of *Candida tropicalis* with biofilms. The first system was the MBECTM assay, and biofilm susceptibility to metals could be discerned using this technique. This model reflects the integrated lifestyle of yeasts in the environment, where planktonic and biofilm forms may grow together in the same locale. However, a limitation to this technique is that the number of cells shed from biofilms is unknown. Thus, the second approach used a well-defined inoculum with an initial number of cells per well of challenge medium equivalent to the biofilm cell density per peg (each of which was placed in a single well of a microtitre plate). These experiments suggested that, relative to planktonic cells, *C. tropicalis* biofilms were less susceptible to metal toxicity.

*Candida tropicalis* 99916 was fastidious with regards to biofilm growth *in vitro*. This microorganism required 2 days of incubation in a rich growth medium (tryptic soy broth) as well as a positively charged substratum. For this latter requirement, the plastic pegs of the MBECTM device were coated with L-lysine prior to biofilm cultivation. Biofilms of *C. tropicalis* could not be grown using Sabaroud dextrose broth, a standard growth medium for yeast (J.J. Harrison, R.J. Turner, and H. Ceri, unpublished data). Although minimal medium formulations designed to reduce inorganic precipitation are desirable for metal susceptibility testing, these could not be used here owing to the laboratory cultivation requirements of *C. tropicalis*.

There are several *in vitro* models that have been used to examine biofilm formation by *C. albicans*. *Candida albicans* grows on surfaces in a series of organized, developmental stages (for a review, see Mukherjee & Chandra, 2004). When grown on polyvinylchloride or elastomer surfaces, *C. albicans* forms an adherent layer of cells that is later covered by hyphal elements and embedded in a matrix of extracellular polymeric substance (EPS) (Chandra et al., 2001a; Kuhn et al., 2002). On denture acrylic, mature *C. albicans* forms an adherent layer of cells that is later covered by hyphal elements and embedded in a matrix of extracellular polymeric substance (EPS) (Chandra et al., 2001a; Kuhn et al., 2002). On the polystyrene surface of the MBECTM device, *C. tropicalis* biofilms formed crests of densely packed cells and/or single-cell layers that later developed into dome-shaped microcolonies. Microcolony formation was
particularly evident by 64 h in our studies (data not shown).
Furthermore, there was some limited differentiation of yeast
cells into hyphal structures near the air–liquid-surface inter-
face of the biofilm under certain test conditions. Candida
tropicalis biofilms were also encased in a layer of EPS, which
is best observed using scanning electron microscopy rather
than CLSM (H. Ceri, D. Fogg, and M. Olson, unpublished
data). The pattern of C. tropicalis 99916 biofilm growth is
thus similar to that reported for C. parapsilosis, which forms
patches of mushroom-shaped communities rather than the
biphasic arrangement of layers seen in C. albicans (Kuhn
et al., 2002).

An advantage to a high-throughput method for metal
susceptibility testing of biofilms is the potential to compare a
number of compounds for microbiological toxicity. Correla-
tive with a previous report examining bacterial biofilm
tolerance to metals (Harrison et al., 2004a), the toxicity of
metal ions belonging to a single periodic group generally
increased with principal quantum number (n). For example
(as shown in Table 2), in group 2B, Hg^{2+} (n = 6, MIC = 1.3 ±
0 mM) was more toxic than Cd^{2+} (n = 5, MIC = 2.8 ±
1.1 mM), which was much more toxic than Zn^{2+} (n = 4,
MIC = 39 ± 0 mM). A similar trend was observed for group
1B, in which Cu^{2+} (n = 4, MIC = 64 ± 0) was less toxic than
Ag^+ (n = 5, MIC = 1.2 ± 0). In this latter case, this trend was
not true of MLCP values, as Cu^{2+} killed C. tropicalis biofilms
whereas Ag^+ did not. However, the lower solubility of Ag^+
in TSB relative to Cu^{2+} is a likely explanation for this exception.
No correlation between MIC, MLCP and MLCB values and
oxidation state or standard reduction potential could be
distinguished. Similar to the case for bacteria (Pseudomonas
aeruginosa, Escherichia coli, and Staphylococcus aureus), Hg^{2+}
and Ag^+ were the two of the most biologically toxic
compounds to C. tropicalis (Harrison et al., 2004a).

Bacterial biofilm tolerance to antimicrobials, including
metals, is currently regarded as a multifactorial phenomenon
(for reviews, see Lewis, 2001; Harrison et al., 2005c). In this
model, there are at least four independent mechanisms at
work: (1) metabolic heterogeneity arising from the restricted
penetration of oxygen and nutrients into the biofilm extra-
cellular polymeric matrix (Walters et al., 2003; Borriello
et al., 2004); (2) a distinct biofilm physiology resulting from
cell-to-cell signalling in the biofilm (Harrison et al., 2004b;
Bjarnsholt et al., 2005); (3) restricted diffusion and/or
penetration of antimicrobials containing charged moieties
into the biofilm matrix (Hall-Stoodley et al., 2004; Harrison
et al., 2005b); and (4) a small subpopulation of persister cells
(Spoering & Lewis, 2001; Keren et al., 2004; Harrison et al.,
2005a,b). The factors contributing to antimicrobial tolerance
in Candida biofilms are largely unknown.

Unlike the case for bacterial biofilms, metabolic stratifica-
tion does not appear to exist in Candida biofilms, because
basal layers of cells still possess high metabolic activity
(Chandra et al., 2001a, b). Neither is there evidence in the
literature that Candida produces specialized persister cells.
However, quorum-sensing in C. albicans biofilms is
mediated by the small molecule farnesol (Hornby et al.,
2001) as well as by the contact-activated kinase Mkc1p,
which signals biofilm development in this dimorphic yeast
(Kumamoto, 2005). Within 1 h of attachment to a substra-
tum, C. albicans has undergone a change in physiological
state that gives elevated resistance to antifungals (Ramage
et al., 2002; Mukherjee & Chandra, 2004). For instance, C.
albicans biofilms have been observed to continue to grow in
high concentrations of fluconazole that were otherwise
inhibitory to planktonic cell growth (Ramage et al., 2002).
The data in this report suggest that a similar phenomenon
may exist to enable C. tropicalis biofilms to resist metal
toxicity. In particular, planktonic cells were killed in a time-
dependent manner by toxic metal cations and oxaymines.
In our assays, this was observed as a decreasing MLCP with
increasing exposure times in broth microdilution assays
performed using a defined inoculum. However (and dissim-
ilar to the case for bacteria), MLCP values discerned using
the MBECTM assay were comparable between 5 and 24 h
exposure times (I.J. Harrison, H. Ceri, and R.J. Turner,
unpublished data). A potential explanation for these obser-
vations is that the biofilms on the pegs may be continuing
to grow, shedding cells into the growth medium in the
presence of metals. In this way, dead and dying planktonic
cells would be continually replenished by the biofilm,
leading to increased MLCP values at prolonged exposures.
A similar trend was observed in our studies for nystatin (I.J.
Harrison, H. Ceri, and R.J. Turner, unpublished data). These
observations were the rationale for the examination of Co^{2+}
and SeO_3^{2−} in greater detail. The data presented in Fig. 3
indicate that C. tropicalis biofilms continued to grow in
concentrations of SeO_3^{2−} (125 mM) that were approxi-
mately twice the concentration that was lethal to planktonic
cells (approximately 50 mM).

This trend is conspicuously different from that in bacte-
rial biofilms. For Pseudomonas aeruginosa and Escherichia
coli, a majority of the population dies at relatively low
concentrations of antibiotics or toxic metals, and a small
‘persister’ cell population survives to high concentrations of
these compounds (Harrison et al., 2005a, b). Strictly speak-
ing, persister cells are not resistant, as they do not grow in
the presence of bactericidal agents. Rather, persisters do not
die and thus exhibit multidrug tolerance (Keren et al., 2004).
Similarly, planktonic cells of Candida exhibit time-depen-
dent tolerance to metals. However, biofilms of C. tropicalis
may continue to grow in the presence of high concentrations
of some antifungals and metals, and thus may be considered
multidrug- and metal-resistant.

A contributing factor to metal resistance in C. tropicalis
biofilms may be sequestration of cations in the extracellular
metal. A previous report has shown that the diffusion of fluconazole and 5-fluorocytosine through Candida biofilms is slowed, requiring 3 to 6 h to equilibrate across these surface-adherent communities (Al-Fattani & Douglas, 2004). However, Candida biofilms exposed for this period of time continued to grow in the presence of these agents, indicating that poor penetration cannot solely account for drug resistance. Similarly, the formation of visible precipitates of metal cations in biofilms suggests that sequestration and limited penetration may be a contributing factor to resistance. However, metal oxyanions could not be precipitated in a similar fashion, indicating that these negatively charged compounds may diffuse across the biofilm matrix. Furthermore, although CrO$_4^{2-}$, AsO$_3^-$ and AsO$_4^{3-}$ killed planktonic C. tropicalis time-dependently, biofilms survived high concentrations of these antimicrobials at the longest exposure time assayed. Together, these data suggest that restricted penetration of metals into the biofilm matrix of C. tropicalis is only one contributing factor for biofilm resistance and/or tolerance.

In this study, we have used C. tropicalis as a model organism for yeasts growing in the environment. The highest concentrations of many metals examined in vitro were not lethal to fungal biofilms. We note that concentrations at the lower end of these in vitro gradients may be similar to those found in some polluted environments (Lopez-Archilla & Amils, 2001; De Vries et al., 2002; Hernández et al., 2003; Schparyk & Parpan, 2004). These same concentrations of metal cations are lethal to some soil bacteria (for example Pseudomonas aeruginosa) (Harrison et al., 2005a, b). This is consistent with reports that yeasts may overwhelm natural systems contaminated with heavy metals (Hagler & Mendocs-Hageler, 1981). Candida spp. may survive bactericidal concentrations of these compounds and may continue growth as biofilms.

Overall, the data suggest that, similar to the case for bacterial biofilms, Candida biofilm resistance is a complex process that relies on more than one mechanism. In our model system, biofilms of C. tropicalis were highly resistant and/or tolerant to a wide range of metal compounds. This suggests that biofilm formation may be an innate means for yeasts to survive metal toxicity in the environment.

**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1.** Candida tropicalis 99916 forms equivalent biofilms in the rows of the MBEC\textsuperscript{TM} \textregistered P\&G assay. Bars indicate the mean and standard deviation of 12 independent replicates for each row of pegs. Biofilm growth was statistically equivalent by means of one-way analysis of variance ($P = 0.402$).

This material is available as part of the online article from http://www.blackwell-synergy.com

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**References**


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