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Short report

Candida auris exhibits resilient biofilm characteristics *in vitro*: implications for environmental persistence

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SUMMARY

Surfaces within healthcare play a key role in the transmission of drug-resistant pathogens. *Candida auris* is an emerging multidrug-resistant yeast which can survive for prolonged periods on environmental surfaces. Here we show that the ability to form cellular aggregates increases survival after 14 days, which coincides with the upregulation of biofilm-associated genes. Additionally, the aggregating strain demonstrated tolerance to clinical concentrations of sodium hypochlorite and remained viable 14 days post treatment. The ability of *C. auris* to adhere to and persist on environmental surfaces emphasizes our need to better understand the biology of this fungal pathogen.

Introduction

Since its discovery in 2009, *Candida auris* has quickly emerged as a prolific nosocomial pathogen, responsible for a number of simultaneous outbreaks globally [1]. It is of considerable interest due to the difficulties associated with identifying and treating this organism and its association with life-threatening infections and high mortality rates.

A key attribute of its pathogenic repertoire is its ability to survive and persist in the environment, yet the methods employed by this multidrug-resistant pathogen to disseminate throughout healthcare environments are still not fully understood. This has profound implications for decontamination and infection control protocols. Therefore, understanding the mechanisms of spread and survival in the hospital environment is critical, particularly as it is able to persist on hospital fomites, extensively colonize individuals, and to survive as biofilms [2,3]. Although traditionally biofilms are associated with formation on an indwelling medical device or on a mucosal substrate, recent investigations have suggested that these communities can facilitate residence and survival upon surfaces within a clinical setting [4].

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Despite the lack of nutrients, these communities adapt to survive and display increased tolerance to both heat and conventional disinfection treatments compared to a free-floating, equivalent cell [5]. *C. auris* has been shown to readily transmit between hospital equipment, such as reusable temperature probes, and patients suggesting limitations of current infection control strategies [4]. Commonly used disinfectants have been shown to be highly effective when tested in suspension, yet our previous data indicate that adherent *C. auris* cells can selectively tolerate biocides, including sodium hypochlorite and peracetic acid, in a substrate-dependent manner [2].

Due to the lack of knowledge concerning survival strategies used by *C. auris*, and the identification of phenotypically distinct morphologies of single-celled and aggregating isolates by Borman and co-workers, we investigated the potential of these phenotypic traits of biofilm formation and cellular aggregation that may aid environmental persistence and survival [6].

Methods

Microbial growth and standardization

Candida auris clinical isolates NCPF 8973 (single cells) and NCPF 8978 (aggregates) were used throughout this study, with phenotypes determined visually using microscopy as previously described [6]. For survival experiments, *Candida glabrata* ATCC 2001 and *Candida parapsilosis* NCPF 8334 were used as reference species. All strains were stored and maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) at 4°C prior to propagation in yeast peptone dextrose (Sigma–Aldrich, Dorset, UK) medium overnight at 30°C. Cells were washed by centrifugation in phosphate-buffered saline (PBS; Sigma–Aldrich, Poole, UK) and standardized to the desired concentration in selected media after counting using a haemocytometer.

Fungal survival assay

To assess the persistence of *Candida* species on dry, non-porous substrates, methods were adapted from Welsh *et al.* with slight modifications [7]. To simulate microbial spillages within the nosocomial environment, various growth conditions were used: PBS, artificial saliva, and 10% fetal calf serum (FCS; Sigma–Aldrich). Cells were grown and standardized as described above to 1×10^8 cells/mL in selected media. Standardized cell suspensions were added to Thermanox™ coverslips (Thermo Fisher Scientific, Loughborough, UK) and after 90 min non-adherent cells and media were removed by washing. Following washing, biomass was subsequently removed from the cover slips via sonication in 1 mL PBS in an ultrasonic water bath (Thermo Fisher Scientific) at 35 kHz for 10 min, defined as day 0. Cells were maintained at ambient temperature for a period of 14 days after initial adherence. Following growth and sonication, biomass was serially diluted for viable cell quantification using the Miles and Misra colony-counting technique.

Fluorescent imaging

For microscopic analysis, dry biofilms were prepared for 14 days as described above. Following incubation, biofilms were stained with FUN-1 dye (20 μM) (Thermo Fisher Scientific). The dye was added to the biofilms and incubated in the dark at 37°C for 30 min. Following staining, biofilms were washed three times with PBS, before images were captured and processed using an EVOS fluorescent microscope (Thermo Fisher Scientific).

Transcriptional analysis

Candida auris cells were grown as described above and RNA was extracted as described previously [8]. In brief, cells were removed from substrates by sonication, before being homogenized using a bead beater and RNA extracted using the TRIzol™ method. Following clean-up with the RNeasy minikit (Qiagen, Crawley, UK), cDNA was synthesized using the High Capacity RNA to cDNA kit (Life Technologies, Paisley, UK) as per the manufacturer's instructions. All primer sequences used for quantitative polymerase chain reaction (qPCR) are shown in Supplementary Table I. The following PCR thermal profiles were used: holding stage at 50°C for 2 min, followed by denaturation stage at 95°C for 10 min and then 40 cycles of 95°C for 3 s and 60°C for 15 s. Expression levels of each gene of interest were calculated using the ΔΔCt method, with expression normalized to the housekeeping gene *ACT*.

Disinfection susceptibility testing

For disinfection experiments, *C. auris* cells were standardized and prepared as described above in 10% FCS. Following the adhesion phase, non-adherent cells were removed through washing with PBS, before substrates were challenged with NaOCl at 1000 ppm (0.1%) for 5 and 10 min or 10,000 ppm (1%) for 5 min, with NaOCl diluted to working concentration in sterile water. Active agents were neutralized with 5% sodium thiosulphate (Thermo Fisher Scientific) for 10 min, which has previously been shown to have no detrimental effects on *C. auris* viability [2]. Viable cells were quantified both immediately (day 0) after neutralization and 14 days after treatment using the colony-counting technique as described above.

Statistical analysis

Data distribution, statistical analysis and graph production was performed using GraphPad Prism (version 8; La Jolla, CA, USA). A Kruskal–Wallis test with post-hoc Dunn's test was used to compare viable cell counts following desiccation. Student's *t*-test was used to compare cell recoveries following treatment. All experiments were performed in triplicate. Differences in means were deemed significant if $P < 0.05$.

Results

To test the theory of biofilm formation being employed as an endurance strategy of *C. auris*, we performed survival studies using two phenotypically distinct isolates based on their ability to form cellular aggregates. Similar to previous findings, *C. auris* was found to remain viable for at least two weeks within a dry environment, regardless of the organic material in which it was suspended (Figure 1A) [7]. It was shown that aggregating cells survived considerably better than their single-cell counterparts in PBS (>2.5 $\log_2$ cfu/mL; $P < 0.001$) and 10% FCS (>4 $\log_2$ cfu/mL; $P < 0.01$). Although not deemed

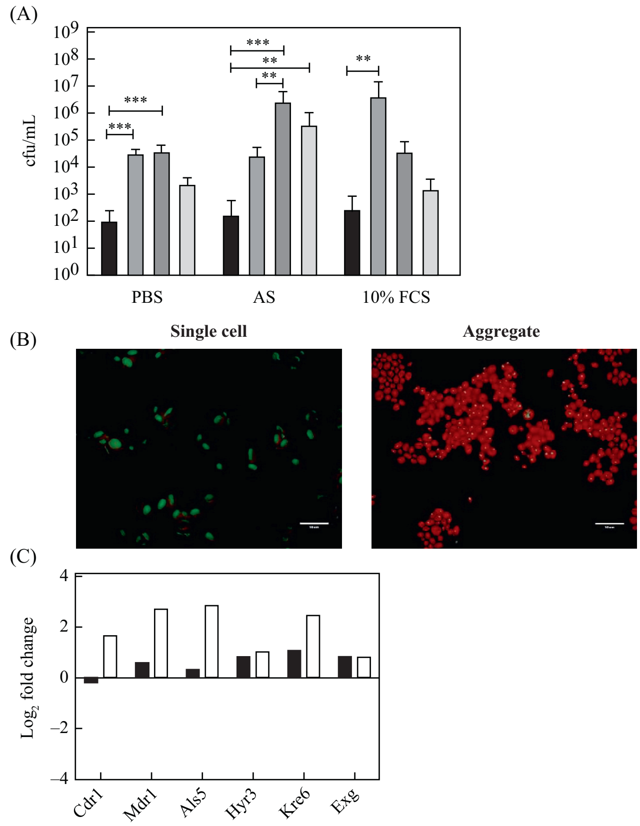

Figure 1. *Candida auris* cellular aggregates display biofilm characteristics to aid environmental survival. The ability of *C. auris* to survive for prolonged periods of time was compared to *Candida glabrata* and *Candida parapsilosis*; $^{**}P < 0.01$, $^{***}P < 0.001$ (A). Viable fungal cells were imaged following staining with FUN-1 dye (B). Gene expression profiles of dry *C. auris* cells on day 0 and day 14 were used to confirm upregulation of biofilm-associated genes following survival (C). Positive fold changes indicate more upregulation in aggregating cells, and genes more highly upregulated in single-celled *C. auris* are represented by negative fold changes. PBS, phosphate-buffered saline; AS, artificial saliva; FCS, fetal calf serum.

statistically significant, the aggregating isolate was shown to recover >2 $\log_2$ cfu/mL more viable cells than the single-celled isolate when suspended in artificial saliva. These findings were reinforced microscopically (Figure 1B), where aggregates of viable *C. auris* cells (red fluorescence) could be seen after 14 days following suspension in 10% FCS, compared to a sparsely populated surface with single cells which were not viable (green fluorescence). When compared to *C. glabrata* and *C. parapsilosis*, the single-celled *C. auris* isolate was shown to yield significantly fewer viable cells than *C. glabrata* in both PBS ($P < 0.001$) and artificial saliva ($P < 0.001$). In addition, recovery of this isolate was also significantly less than that of *C. parapsilosis* in artificial saliva ($P < 0.01$). The aggregative *C. auris* isolate also yielded significantly fewer viable cells than *C. glabrata* in artificial saliva ($P < 0.01$); however, $>1\times10^4$ cfu/mL were recovered.

Due to these observed differences between single-celled and aggregating strains of *C. auris*, we then assessed the potential role of biofilm characteristics in accounting for these findings. Using transcriptional analysis of a panel of biofilm-associated genes including genes involved in drug resistance (*Cdr1* and *Mdr2*), adhesion (*Als5* and *Hyr3*) and extracellular matrix (*Kre6* and *Exg*) were shown to be upregulated in the aggregating *C. auris* phenotype (Figure 1C). Following 14 days of starvation, both drug resistance genes were upregulated by 1.6 and 2.6 $\log_2$-fold change (*Cdr1* and *Mdr1* respectively) in the aggregative *C. auris* strain. In addition, the adhesin *Als5* (2.8 $\log_2$-fold change) and the glucan production protein *Kre6* (2.4 $\log_2$-fold change) demonstrated increased expression in the aggregating strain compared to the single-celled isolate.

Due to the propensity of *C. auris* to survive for prolonged periods, we next tested the survival ability of the organism post disinfection treatment. Quantification immediately after NaOCl treatment revealed that viable *C. auris* cells were recovered regardless of exposure time or concentration, with the aggregating strain consistently yielding significantly more viable quantities irrespective of treatment condition (Figure 2). Interestingly, despite previous exposure to 1000 ppm NaOCl for 5 min, $>1\times10^3$ cfu/mL of aggregating cells were recovered 14 days after treatment, compared to no recovery of viable single-celled equivalents (Figure 2A). However, following an increase in exposure time to 10 min (Figure 2B) or increase in NaOCl concentration to 10,000 ppm (Figure 2C), no viable *C. auris* cells were detected following 14 days of incubation.

Discussion

Micro-organisms employ various survival strategies to adapt and aid persistence in various ecological niches, enhancing the likelihood of effectively establishing transmission of infection. Here we show that cellular aggregation and expression of biofilm-like characteristics of *C. auris* can facilitate prolonged survival after disinfection processes. Biofilm formation is typically associated with treatment failure and the recurrence of chronic infections; however, recent studies have suggested that it may also be employed as an environmental survival strategy of nosocomial pathogens. It has been previously shown that *C. auris* can survive and persist on various substrates including steel and plastic for up to four weeks [7]. In accordance with previous studies, we have demonstrated that viable *C. auris* cells can be recovered 14 days after inoculation across several biologically relevant soiling agents. The aggregating strain of *C. auris* was shown to have comparable survival properties to *C. glabrata* and *C. parapsilosis* in PBS, with *C. glabrata* recovering more viable cells in artificial saliva, likely because *C. glabrata* is a commensal of the oral microbiota.

The aggregation phenomena were first described by Borman *et al.*, and were shown to be to less virulent *in vivo* in comparison to a single-celled isolate, likely due to an inability of these isolates to disseminate in host [6]. These phenotypes were initially hypothesized to be related with their associated genetic clade, but more recently the ability to aggregate has been shown to be an inducible trait, with exposure to triazoles and echinocandins triggering single-celled isolates to form aggregates [9]. Although it is not as virulent, we have shown that an aggregative isolate has an enhanced survival capacity compared to a single-celled isolate and can continually persist for at least two weeks after exposure to clinical concentrations

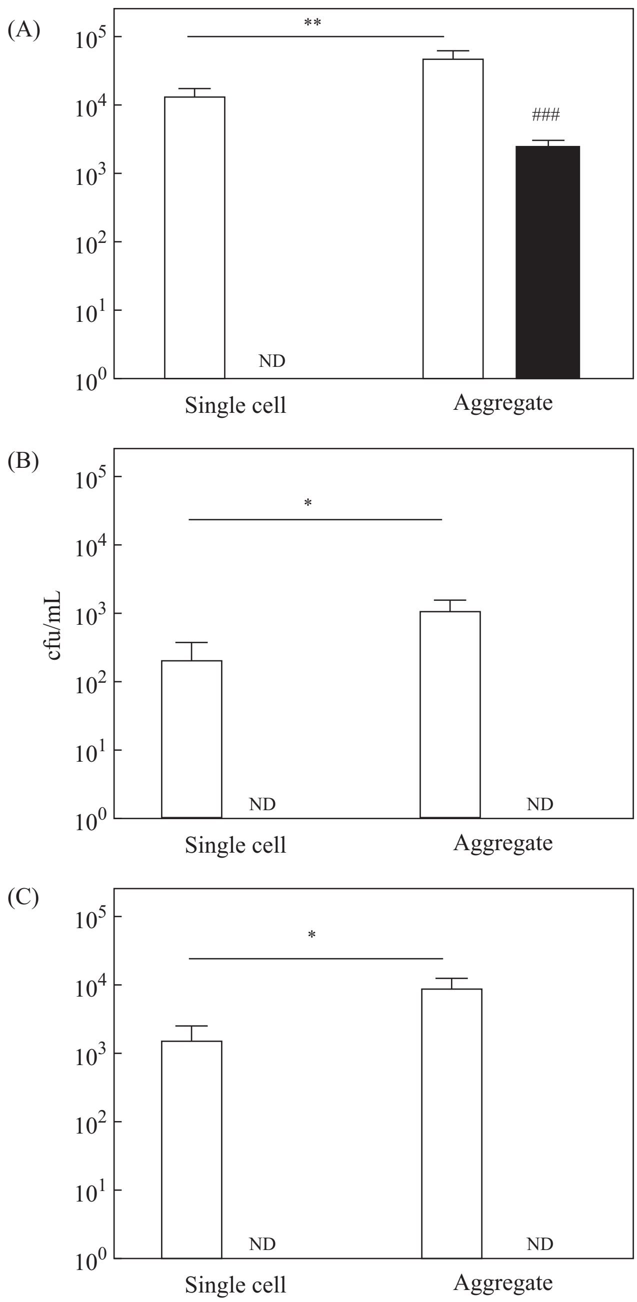

Figure 2. Cellular aggregates of *Candida auris* can survive for prolonged periods following NaOCl disinfection. Survival was also monitored after a 14-day period following disinfection challenge with NaOCl. Viable cells of aggregating and single-celled *C. auris* strains were enumerated by colony-forming unit quantification following treatments with NaOCl at 1000 ppm for 5 (A) and 10 min (B) and at 10,000 ppm for 5 min (C). Experiments were performed in triplicate on three separate occasions (*$P < 0.05$, **$P < 0.01$ comparing day 0; $^{###}P < 0.001$ comparing day 14). ND, not detected.

of NaOCl. The effect of the reversible switch in *C. auris* with regards to disinfection remains unknown, though it might be speculated that induction of aggregate formation from the single-celled phenotype could be employed as a mechanism to facilitate environmental survival.

A recent study from Ledwoch and Maillard assessed the ability of a *C. auris* dry biofilm to withstand a panel of different disinfectants, such as peracetic acid and chlorine dioxide [10]. In support with this and our previous study, they showed that adherent *C. auris* cells could selectively tolerate various different biocides, as well as significant levels of transferability to new, sterile surfaces post treatment [2]. The authors' model used the *C. auris* type strain DSMZ 20192, which, using traditional methodologies, produces single cells, minimal levels of biofilm biomass, and is susceptible to fluconazole (unpublished data). Therefore, it could be speculated that clinical isolates of this organism, which can form more robust biofilms and have the capacity to aggregate, may have enhanced implications for both tolerance and transferability to treatment.

To confirm a role for biofilms in facilitating environmental persistence, a panel of biofilm associated genes, selected according to our group's previous transcriptional characterization of *C. auris* biofilms, was assessed [8]. These genes were highly expressed across both phenotypes; however, comparative analysis revealed increased expression of approximately two-fold of several of these genes, which are involved in adhesion, extracellular matrix (ECM) production, and efflux pumps. ECM production is a well-documented resistance mechanism in pathogenic fungal biofilms of *Candida* spp. [8]. Increasing ECM production could provide the necessary protection for *C. auris* to survive extended periods of desiccation and retain viability following terminal disinfection.

In conclusion, this study reveals a survival mechanism employed by this emerging pathogenic yeast that can facilitate its environmental persistence, even after being challenged with NaOCl. As we have previously suggested, the length of exposure to NaOCl is an important consideration, with increased exposure appearing to be more efficacious than an increased concentration [2]. Further studies understanding the underlying biology associated with the aggregative phenotype and dry surface biofilms will allow the development of more effective infection, prevention, and control measures to control *C. auris* within the nosocomial environment.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

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