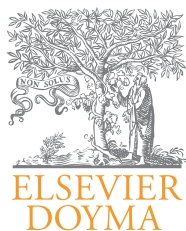Passerini de Ross, Beatriz; García, Carlos; Alcaraz, Eliana; Franco, Mirta

Stenotrophomonas maltophilia interferes via the DSF-mediated quorum sensing system with Candida albicans filamentation and its planktonic and biofilm modes of growth

REVISTA ARGENTINA DE MICROBIOLOGÍA

ORIGINAL ARTICLE

Stenotrophomonas maltophilia interferes via the DSF-mediated quorum sensing system with *Candida albicans* filamentation and its planktonic and biofilm modes of growth

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Abstract
Stenotrophomonas maltophilia is a nosocomial pathogen of increasing importance. *S. maltophilia* K279a genome encodes a diffusible signal factor (DSF) dependent quorum sensing (QS) system that was first identified in *Xanthomonas campestris* pv. *campestris*. DSF from *X. campestris* is a homologue of farnesoic acid, a *Candida albicans* QS signal which inhibits the yeast-to-hyphal shift. Here we describe the antagonistic effects of *S. maltophilia* on *C. albicans* on filamentation as well as on its planktonic and biofilm modes of growth. To determine the role of the DSF-mediated quorum sensing system in these effects, *C. albicans* ATCC 10231 and *C. albicans tup1* mutant, locked in the filamentous form, were grown with K279a or with its *rpfF* deletion mutant (DSF⁻). A significant reduction in viable counts of *C. albicans* was observed in planktonic cocultures with K279a as well as in mixed biofilms. Furthermore, no viable cells of *C. albicans tup1* were recovered from K279a mixed biofilms. Fungal viability was also assessed by labeling biofilms with SYTO 9 and propidium iodide. Confocal images showed that K279a can kill hyphae and also yeast cells. Light microscopic analysis showed that K279a severely affects hyphae integrity. On the other hand, the presence of K279a *rpfF* did not affect fungal morphology or viability. In conclusion, we report for the first time that *S. maltophilia* interferes with two key virulence factors of *C. albicans*, the yeast-to-hyphal transition and biofilm formation. DSF could be directly responsible for these effects or may induce the gene expression involved in antifungal activity.

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Stenotrophomonas maltophilia interfiere a través del sistema de "quorum sensing" mediado por DSF con la filamentación y el crecimiento planctónico y en biofilm de *Candida albicans*

Resumen

Stenotrophomonas maltophilia es un patógeno nosocomial de importancia creciente. El genoma de *S. maltophilia* K279a codifica un factor de señalización difusible (DSF), auto-inductor de "quorum sensing" (QS), identificado previamente en *Xanthomonas campestris* pv. *campestris*. El DSF de *X. campestris* es homólogo del ácido farnesoico, señal de QS de *Candida albicans*, que inhibe la transición levadura-hifa. En este trabajo se describe el efecto antagónico de *S. maltophilia* sobre la filamentación y el crecimiento planctónico y en biofilms de *C. albicans*. Para determinar la participación del sistema de QS mediado por el DSF en dichos efectos, *C. albicans* ATCC 10231 y la mutante *C. albicans tup1*, que solo crece en forma filamentosa, fueron cultivadas en presencia de K279a o de su mutante K279a *rpfF* (DSF⁻). Se observó una reducción significativa del número de viables de *C. albicans* en cultivos planctónicos y biofilms desarrollados en presencia de K279a. Es de señalar que no se recuperaron células viables de *C. albicans tup1* a partir de biofilms mixtos en presencia de K279a. Las imágenes de microscopía confocal revelaron que K279a produce la muerte de hifas y levaduras en biofilms mixtos teñidos con ioduro de propidio y SYTO 9. El análisis por microscopía óptica mostró que K279a afecta la integridad de las hifas. En cambio, la presencia de K279a *rpfF* no afectó la morfología ni la viabilidad fúngica. En conclusión, informamos por primera vez que *S. maltophilia* interfiere con dos factores de virulencia de *C. albicans*, la transición levadura-hifa y la formación de biofilms. Estos efectos pueden ser mediados por el DSF en forma directa o a través de la inducción de genes involucrados en la actividad antifúngica.
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Introduction

Stenotrophomonas maltophilia (formerly *Pseudomonas maltophilia* and *Xanthomonas maltophilia*) is a widespread environmental, multidrug resistant bacterium. It has become a nosocomial pathogen of increasing importance; in fact, it is the third most common nosocomial non-fermenting gram-negative bacterium[7]. Infection occurs principally in immunocompromised subjects, and in patients exposed to invasive devices and/or broad spectrum antibiotics[5,21]. *S. maltophilia* has also emerged as one of the most common bacteria isolated from the airway of cystic fibrosis (CF) patients[28,34], nevertheless, there is controversy about the role of this opportunistic pathogen as causative agent of CF.

An important virulence factor of *S. maltophilia* is the capacity to form biofilms, communities of microbial cells that grow on biotic or abiotic surfaces embedded within exopolymeric material[16,24,27]. Microorganisms growing in biofilms exhibit phenotypic characteristics that are distinct from those of their planktonic counterparts, including increased resistance to host immune defences and antimicrobial compounds[9]. Microorganisms communicate using chemical signals (autoinducers) to sense cell density and coordinate their physiological processes, including biofilm formation, in a process known as quorum sensing (QS). QS also plays an important role in interspecies communication, including interactions between prokaryotes and eukaryotes[30,33].

The genome of *S. maltophilia* K279a encodes a diffusible signal factor (DSF) dependent QS system that was first identified in *Xanthomonas campestris* pv. *campestris*[11,17]. DSF synthesis is completely dependent on *rpfF*, which is part of the *rpf* operon (for regulation of pathogenicity factors)[2]. The cross-kingdom antagonistic activity of DSF (*cis*-11-methyl-2-dodecenoic acid) from *X. campestris* has been reported on *Candida albicans* germ tube formation[35].

C. albicans, one of the most important opportunistic human fungal pathogen, causes various diseases ranging from superficial mucosal infections to life-threatening systemic infections[6]. The ability of *C. albicans* to undergo morphogenic shift from yeast to filamentous morphology is central to its pathogenesis and biofilm formation[29,32]. This morphological transition is induced *in vivo* and *in vitro*. Host factors or environmental factors, such as mammalian serum and high temperatures (37 °C), are required for *C. albicans* morphogenesis. Two related QS compounds, farnesoic acid and farnesol inhibit the yeast-to-hyphal shift. *C. albicans* ATCC10231 produces farnesoic acid while a number of *C. albicans* clinical isolates produce farnesol[15,23]. Furthermore, it has been reported that farnesol addition can block *C. albicans* biofilm formation[29]. The DSF family of QS signals has been implicated in the interkingdom signaling between bacteria and *C. albicans*[30,33]. DSF from *X. campestris* is a structural and functional homologue of farnesoic acid, and it has been suggested that the bacterial QS signal is recognized by the receptor of farnesoic acid, leading to an

arrest in filamentation[35]. Furthermore, *cis*-2-dodecenoic acid (BDSF) from *Burkholderia cenocepacia* inhibits *C. albicans* germ tube formation either as a pure compound or in coculture, and BDSF also blocks *C. albicans* biofilm formation by interfering with the morphological switch[3,36].

In the present work, we report the antagonistic effects of *S. maltophilia* K279a on *C. albicans* filamentation as well as on its planktonic and biofilm modes of growth. To determine the role of the DSF-mediated quorum sensing system in these effects, *C. albicans* was grown with *S. maltophilia* K279a or its *rpfF* deletion mutant (DSF⁻). *C. albicans* ATCC 10231 was used as a wild type strain while *C. albicans* tup1 mutant, locked in the filamentous form, was used to investigate the interactions between bacterial strains and *C. albicans* filaments.

Materials and methods

Strains and culture conditions

S. maltophilia K279a, whose genome is fully sequenced (http://www.sanger.ac.uk/Projects/S_maltophilia/)[1,7], and its derivative DSF-deficient mutant K279a *rpfF*[11] were a kind gift from Dr. M. Dow (BIOMERIT Research Centre, Ireland). *C. albicans* ATCC10231 was used in this study as a wild type strain. The mutant *C. albicans* tup1[4], unable to form yeasts, was a gift from Dr. R. Kolter (Harvard Medical School, Boston, USA). Bacterial strains were routinely cultured at 37 °C on trypteine soy agar (TSA, Britania, Argentina) while *C. albicans* strains were grown on Sabouraud dextrose agar (SDA, Britania). Unless otherwise stated, all experiments were performed with microorganisms grown in trypteine soy broth (TSB, Britania) supplemented with 0.2% (w/v) dextrose (TSB-d), incubated at 200 rpm on a gyratory water bath shaker (Model G75, New Brunswick Scientific Co. Edison N J, USA), at either 30 °C or 37 °C, depending on the experiment. We had previously confirmed that TSB-d enhanced the yeast-to-hyphae transition and supported *C. albicans* biofilm formation even though it was slightly less effective than RPMI 1640, a medium generally chosen for filamentation and biofilm assays[26]. TSB-d was chosen because this medium supported *S. maltophilia* growth (data not shown) better than RPMI 1640. The viability of *C. albicans* was determined by plating on SDA or SDA supplemented with 100 μg/ml ciprofloxacin (ICN, Basingstoke, UK) to suppress the growth of *S. maltophilia*. When required, fetal bovine serum (FBS, Bioser, Argentina) was added to TSB-d at a final concentration of 5%. When appropriate, 4.7 mM DSF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) stock solutions were made in ethanol and then diluted in TSB-d to the required concentration (20-200 μM). An equal volume of ethanol was used in all appropriate controls.

Inhibition of *C. albicans* germ tube formation by *S. maltophilia*

Analysis of *C. albicans* morphology was performed as described previously with modifications[3]. Briefly, *C. albicans* ATCC 10231 was grown as yeast cultures overnight at 30 °C

in TSB-d. *S. maltophilia* strains were grown under the same conditions but at 37 °C. Overnight *C. albicans* cultures were diluted in 2 ml of fresh TSB-d to contain approximately 10^5 CFU/ml. Coculture experiments were performed by adding K279a or K279a *rpfF* cultures at a final concentration of 10^6 CFU/ml (ratio bacteria-fungi of 10:1). After 15 h of incubation at 30 °C, FBS was added and incubation proceeded for 3 h at 37 °C for induction of germ tube formation. Then, cells were Gram stained and percentages of germinal tube production were examined by light microscopy (Olympus BX50, 400X magnification), counting about 400 *C. albicans* cells per sample.

Inhibition of *C. albicans* filamentation by DSF

Overnight cultures of *C. albicans* ATCC 10231 in TSB-d at 30 °C were diluted 10-fold in fresh TSB-d supplemented with FBS and DSF (0-200 μM). After 3 h incubation at 37 °C, percentages of germ tube formation were examined as described above.

Survival of *C. albicans* in the presence of *S. maltophilia* in planktonic cultures

C. albicans survival assays were performed as described previously with modifications[13]. Briefly, fungal (*C. albicans* ATCC10231 or *C. albicans* tup1) and bacterial strains (K279a or the *rpfF* mutant) were grown in TSB-d at 37 °C. Overnight cultures were diluted in 2 ml of fresh TSB-d and standardized as described above. Fungal cultures used as controls and cocultures with bacterial strains (ratio bacteria-fungi of 10:1) were grown for 48 and 72 h at 37 °C. After incubation, the number of viable fungal cells, in the presence or absence of *S. maltophilia* strains, was estimated by plating 10-fold serial dilutions of these suspensions. After 24 h of incubation at 37 °C, colony-forming units were counted and converted to $\log_{10}$ CFU/ml. Furthermore, cells were Gram stained and examined by light microscopy (400× magnification). Microphotography was done with an Olympus DP70 digital camera. The same experiment was also performed with *C. albicans* ATCC10231 and bacterial strains but cultures were incubated at 30 °C, a condition that favors yeast growth form.

C. albicans biofilm formation in the presence of *S. maltophilia*

Biofilms were prepared using a static microtiter plate model as described previously[25]. *C. albicans* ATCC 10231 or *C. albicans* tup1 overnight cultures grown in TSB-d at 37 °C were standardized to contain approximately 10^6 CFU/ml, and for mixed biofilms, bacterial cultures were added in the ratio of 1:1. For each test condition, 4 wells of a sterile flat-bottom 96-well polystyrene microtiter plate (TPP, Trasandingen, Switzerland) were filled with 200 μl of the standardized inocula. Uninoculated medium controls were included. After static incubation for 72 h at 37 °C, the culture medium was removed from each well and plates were washed twice with sterile PBS to remove non-adherent cells. The surfaces of the wells were scraped with sterile

cotton swabs (4 wells per condition). Swabs transferred into tubes containing 2 ml of PBS were sonicated (ELMA Transonic TI-H-5, Germany) for 3 min and vortexed vigorously to aid the dissolution of microrganism clumps. The viability of *C. albicans* biofilms, in the presence or absence of *S. maltophilia* strains, was determined by plate counts as described above. The limit of detection in our experimental conditions was 40 CFU/ml. A fluorescence-based technique that distinguishes live cells from dead ones based on the presence of an intact cytoplasmic membrane was also used. Biofilms used for confocal scanning laser microscopy (CSLM) were developed on 8-well chamber slides (Nunc Lab-Tek, Rochester, NY, USA) containing a borosilicate glass base. Chambers were filled with 400 μl of the standardized inocula. After 72 h of static incubation at 37 °C, the wells were rinsed with sterile normal saline [NS; 0.9% (w/v) NaCl] and refilled with NS containing 2.5 μM SYTO 9 (Molecular Probes, Grand Island, NY, USA) and 15 μM propidium iodide (PI, Sigma, St. Louis, Missouri, USA). Live SYTO 9-stained cells and dead propidium iodide-stained cells were visualized with a confocal laser-scanning microscope (Carl Zeiss LSM510-Axiovert 100M) after 20 min incubation in the dark. Dual-colour images were acquired by sequentially scanning with a 488 nm argon laser and emitted fluorescence was recorded within the range 505-530 nm or 530-600 nm in order to visualize SYTO 9 or PI fluorescence, respectively. Images were reconstructed using the ZEN 2009 light edition. CSLM was performed twice independently. Each time, two wells were used per condition. Lastly, biofilms used for light microscopy were formed on Thermanox cover slips placed in 12 well microtiter plates under the same conditions. After incubation, cover slips were removed, washed with PBS, Gram stained and evaluated by light microscopy at 400× magnification.

Statistical analysis

All assays were carried out at least in triplicate and repeated three times, except CLSM. Results were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni´s multiple-comparison test (GraphPad Prism version 5.00 for Windows, Graph Pad Software, San Diego, CA, USA). Differences were considered significant at a *p* value of <0.05.

Results and discussion

Inhibition of *C. albicans* germ tube formation

The microscopic analysis showed that approximately 61% of *C. albicans* ATCC 10231 cells counted formed germ tubes when cocultured with K279a *rpfF*, a similar percentage to that observed in the control (63%). On the other hand, the coculture with K279a significantly decreased germ tube formation to approximately 41% (*p* <0.001) (Fig. 1A). Thus, the inhibitory effect of *S. maltophilia* was dependent on the production of DSF, which could be recognized by the receptor of farnesoic acid leading to an arrest in filamentation as it was suggested for DSF from *X. campestris*[35]. Furthermore, the inhibitory activity of
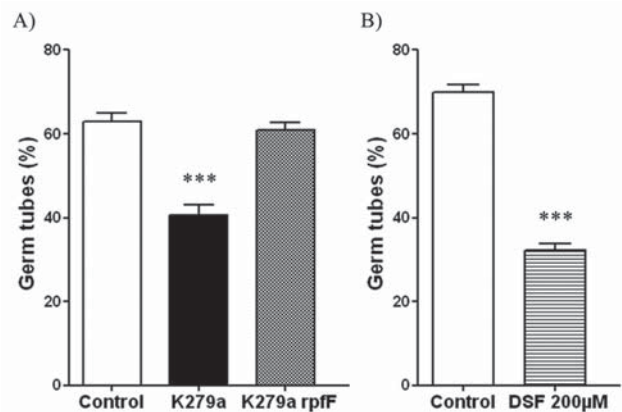

Figure 1 Inhibition of *C. albicans* germ tube formation. A) Effect of *S. maltophilia* on *C. albicans* ATCC 10231 germ tube formation. B) Effect of commercial DSF on *C. albicans* ATCC 10231 germ tube formation. A and B) cells were Gram stained and percentages of germinal tube production were examined by light microscopy, counting about 400 *C. albicans* cells per sample. The mean and standard error from three repeats are presented. ***Significant difference (*p* <0.001) respect to the control.

commercial DSF against *C. albicans* ATCC 10231 germ tube formation was also evaluated in the presence of FBS. After induction at 37 °C for 3 h, the microscopic analysis showed that more than 70% of the cells formed germ tubes in the control. DSF at a final concentration of 200 μM caused a marked reduction in germ tube formation of approximately 32% (*p* < 0.001) (Fig. 1B). However, at lower concentrations, DSF did not significantly inhibited germ tube formation under these conditions (data not shown).

Cross-kingdom antagonistic activity of the DSF family has been reported in *C. albicans* filamentation[30]. BDSF from *B. cenocepacia* is the most potent inhibitor of *C. albicans* germ tube formation, followed by DSF from *X. campestris*[3,35]. Our results highlighted the role of DSF from *S. maltophilia*, directly or via the induction of genes involved in antifungal activity, in the inhibition of filamentation. Other autoinducers, including homoserine lactones (HSL) such as 3-oxo-C_{12}-HSL produced by *Pseudomonas aeruginosa*, are able to inhibit the *Candida* yeast-to-hypha transition[14]. However, it is not known if all these molecules exert their action through the same mechanism. Farnesol can affect more than one regulatory system in different ways. It was demonstrated that farnesol action involves the trans-criptional repressor Tup1 that negatively regulates the yeast-to-hypha transition[19]. Recently, it has been shown that farnesol and 3-oxo-C_{12}-HSL directly inhibit the adenylyl cyclase Cyr1p, while dodecanol prevents cAMP-dependent hyphal development through a process dependent upon the transcriptional repressor SFL1[12]. Furthermore, *B. cenocepacia* and possibly BDSF inhibit *C. albicans* hyphal development through an SFL1-dependent signaling pathway similar to that of dodecanol, which is not a physiologically relevant QS molecule of *C. albicans*[12]. Further studies are required to uncover the molecular mechanisms of DSF and BDSF in *C. albicans* morphogenesis.

Survival of *C. albicans* in the presence of *S. maltophilia* in planktonic cultures

Figure 2 shows *C. albicans* ATCC 10231 and *C. albicans tup1* survival assays performed at 37 °C. There were not statistically significant differences between the number of viable fungal cells in *C. albicans* ATCC 10231 cultures and cocultures with K279a *rpfF*, either after 48 h or 72 h of incubation (Fig. 2A). On the other hand, cocultures with K279a showed a different behavior. After 48 h of incubation, the number of viable fungal cells was similar to that of the control (7.70 ± 0.24 $\log_{10}$ CFU/ml and 8.18 ± 0.39 $\log_{10}$ CFU/ml, respectively). However, after 72 h the number of viable cells declined significantly by 2 logs (p <0.001) (Fig. 2A). Survival assays were also performed under a condition that favors yeast growth by incubation of *C. albicans* ATCC 10231 and bacterial strains at 30 °C. Interestingly, under this condition K279a did not affect *C. albicans* viability. After 72 h of incubation the number of viable fungal cells was similar to that of the control (8.08 ± 0.44 $\log_{10}$ CFU/ml and 8.25 ± 0.27 $\log_{10}$ CFU/ml, respectively).

It was observed that the number of viable cells of *C. albicans* wild type control was similar after 48 h and 72 h of incubation (Fig. 2A), but for *C. albicans tup1* control, a decreased of 1 log was observed after 72 h (8.47 ± 0.11 $\log_{10}$ CFU/mland 7.46 ± 0.09 $\log_{10}$ CFU/ml, respectively) (Fig. 2B). Cocultures of the *tup1* mutant and K279a *rpfF* showed a similar behavior to that observed in the *tup1* control. Moreover, *C. albicans tup1* in association with K279a showed a significant reduction in the number of viable fungal cells by 2.5 logs after 72 h of incubation (p <0.001) (Fig. 2B).

The effect of *S. maltophilia* strains on *C. albicans* was also evaluated by light microscopy. The morphology of the Gram stained cells after 72 h of incubation is illustrated in Figure 3. *C. albicans* wild type cultures presented yeast and hyphae-cells (Fig. 3A). Cocultures with K279a *rpfF* showed bacteria associated to fungal cells (Fig. 3B).On the other

hand, in the presence of K279a, hyphae-cells but not yeast-cells, showed a damaged aspect (Fig. 3C). Microscopic observation of *C. albicans tup1* control and coculture with K279a *rpfF* revealed unaffected hyphae structures (Figs. 3D and 3E). However, damaged hyphae-cells with discolored areas and apparently altered walls were observed in the presence of K279a (Fig. 3F).The described effects of K279a were less evident at 48 h than at 72 h of incubation at 37 °C (data not shown).

The above results show that the antagonistic effect of *S. maltophilia* K279a on *C. albicans* survival was dependent on DSF production. It is worth mentioning that K279a did not affect *C. albicans* ATCC 10231 viability in coculture experiments performed at 30 °C, a condition that favors yeast growth form. We have previously observed that *S. maltophilia* K279a cultures incubated at 30 °C or 37 °C for 72 h achieved similar final culture densities and presented DSF activity (data not shown).

In fact, the microscopic analysis showed that K279a affects *C. albicans* hyphae-cells, probably by the action of virulence factors whose synthesis is DSF -dependent. Fouhy *et al.*[11] reported that DSF signaling regulates protease production, a virulence factor of *S. maltophilia*. Furthermore, these authors showed in a nematode model that K279a *rpfF* reduced killing activity compared to the wildtype K279a. In our study, the prolonged time (72 h) needed to detect the detrimental effects of K279a in *C. albicans* viability and the association observed microscopically between bacterial and fungal cells suggest that the adherence of bacteria to fungal cells could facilitate *S. maltophilia* virulence factor action.

C. albicans biofilm formation in the presence of *S. maltophilia*

C. albicans biofilms were first studied using a static microtiter plate model in the presence or absence of
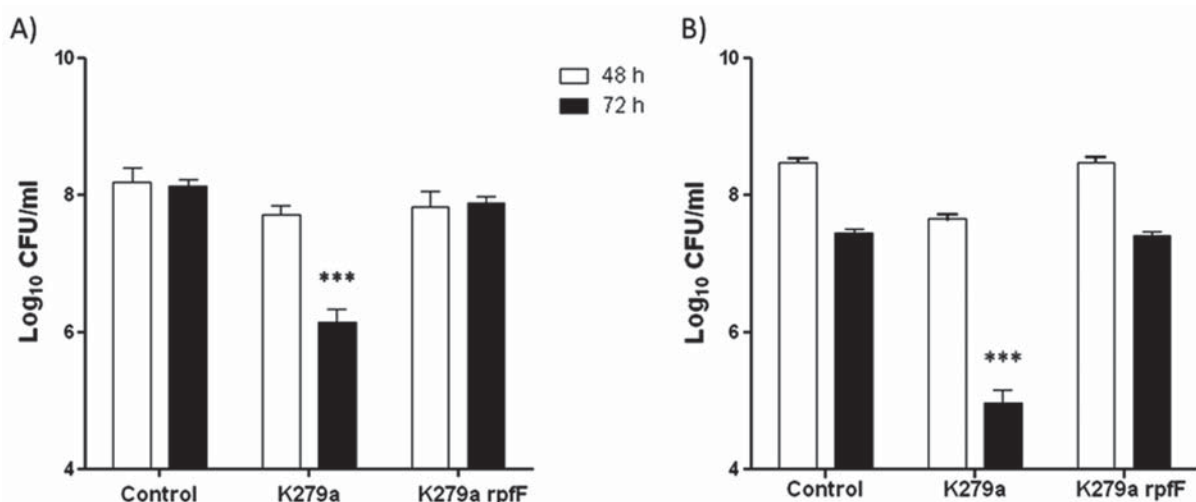

Figure 2 Survival of *C. albicans* in the presence of *S. maltophilia*. The viability of *C. albicans* ATCC 10231 (A), and the constitutively filamentous *C. albicans tup1* mutant (B) was measured in the presence or absence of *S. maltophilia* strains. Cultures were grown in TSB-d for 48 h and 72 h at 37 °C and viable counts were performed on SDA or SDA supplemented with ciprofloxacin. Each bar represents mean ± SD $\log_{10}$ CFU/ml from three independent assays. ***Significant difference (p <0.001) with respect to the control at the same time of incubation.
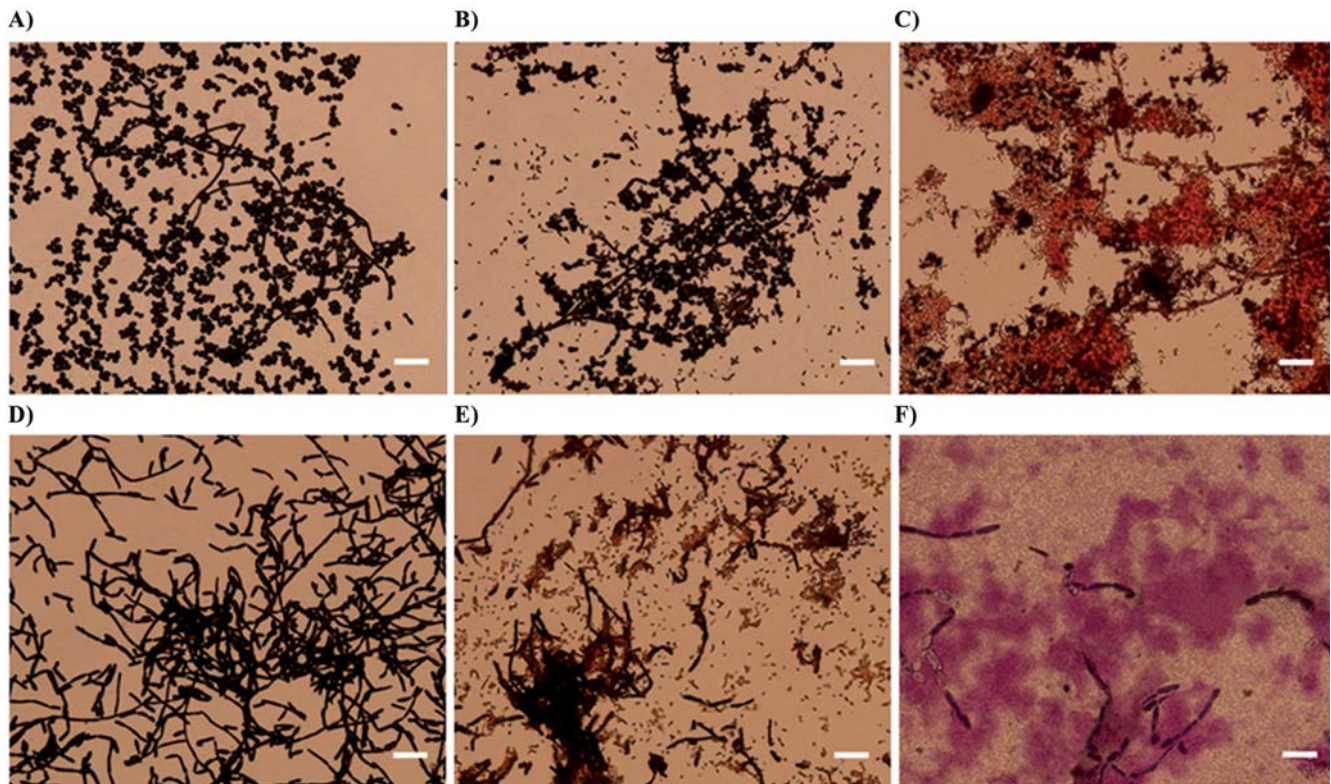
Figure 3 Microscopic analysis of planktonic cultures of *C. albicans* in the presence of *S. maltophilia*. *C. albicans* ATCC 10231 (upper panel) and *C. albicans tup1* (lower panel) were grown alone or in the presence of bacterial strains in TSB-d for 72 h at 37 °C. (A) and (D) controls, (B) and (E) cocultures with K279a *rpfF*, and (C) and (F) cocultures with K279a. Cells were Gram stained and examined by light microscopy (400× magnification). Scale bars correspond to 10 μm.

S. maltophilia strains. After 72 h of incubation, fungal biofilm formation was evaluated by viable counts (Fig. 4). Biofilms of *C. albicans* ATCC 10231 and *C. albicans tup1* formed in the presence of K279a *rpfF* showed a number of fungal cells similar to that observed in the respective controls. Furthermore, in the presence of K279a the count of *C. albicans* ATCC 10231 cells decreased by 3 logs with respect to the control ($p < 0.001$) (Fig. 4). The effect of K279a on mutant *tup1* biofilms was more severe, and viable counts were under the limit of detection (Fig. 4).

Fungal viability was also assessed by simultaneous labeling biofilms developed on glass chamber slides with the green-fluorescent SYTO9 dye and the red-fluorescent nucleic acid stain PI. Confocal images acquired from *C. albicans* ATCC 10231 or *C. albicans tup1* biofilms grown in mixed cultures with K279a *rpfF* showed that most of the fungal cells were alive (Figs. 5A and 5C). In contrast, fungal cell death occurred in *C. albicans* wild type or mutant biofilms developed in the presence of K279a. Furthermore, amorphous red PI-stained material, which may represent DNA containing debris from lyzed cells, was also observed (Figs. 5B and 5D). The images showed that K279a can kill hyphae and also yeasts since *C. albicans* wild type biofilms mainly included yeast cells under this study conditions.

To further study the effect of *S. maltophilia* on fungal sessile cultures, biofilms formed on Thermanox cover slips were Gram stained and evaluated by light microscopy. Control biofilms of *C. albicans* wild type mainly included
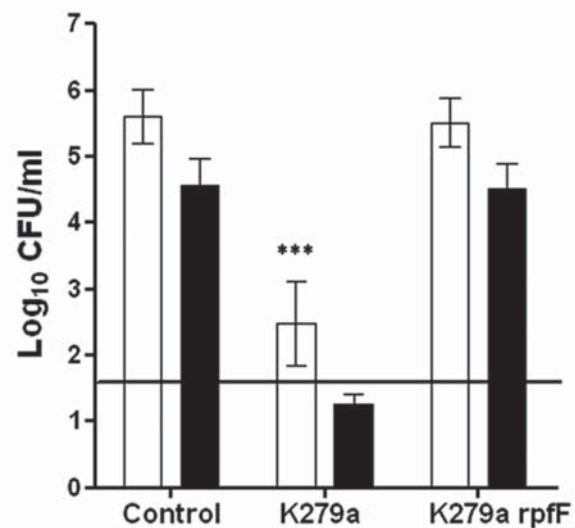

Figure 4 Evaluation of the viability of *C. albicans* 72 h biofilms in the presence of *S. maltophilia*. The viability of *C. albicans* ATCC 10231 (white bars) or *C. albicans tup1* (black bars) biofilms formed in microtiter plates was determined by plate counts performed on SDA or SDA supplemented with ciprofloxacin. The limit of detection was 40 CFU/ml and is represented by a horizontal black line. Each bar represents mean ± SD $\log_{10}$ CFU/ml from three independent assays. ***Significant difference ($p < 0.001$) with respect to the control.
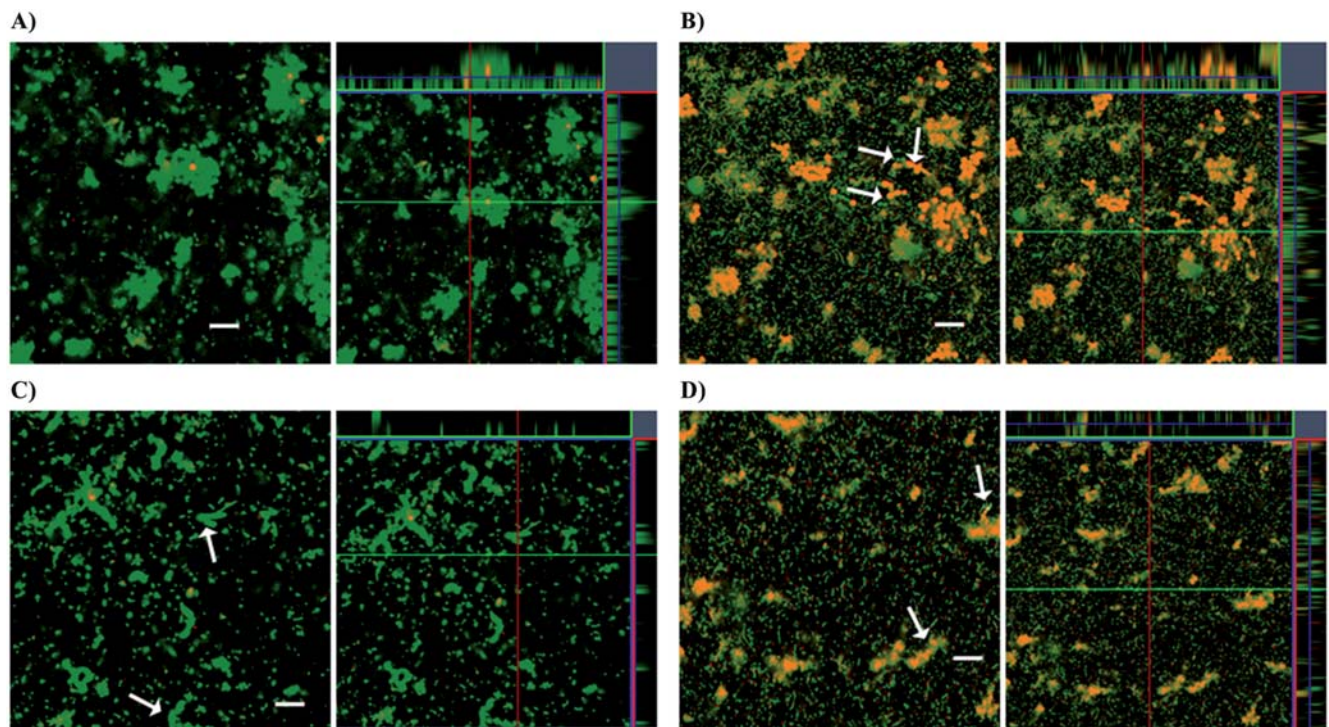
Figure 5 Confocal scanning laser microscopy images of *C. albicans* biofilms in the presence of *S. maltophilia*. *C. albicans* ATCC 10231 (upper panel) and *C. albicans tup1* (lower panel) biofilms were formed on 8-well chamber slides in the presence of bacterial strains. (A) and (C) cocultures with K279a *rpfF*, and (B) and (D) cocultures with K279a. After 72 h of incubation at 37 °C biofilms were stained for cell viability. Live SYTO 9-stained cells (green) and dead propidium iodide-stained cells (red-orange) were visualized with a CSLM (400× magnification). For each micrograph, the middle panel represents the *x-y* plane, and the adjacent top and side panels represent the *x-z* and *y-z* planes, respectively. Scale bars correspond to 20 μm. Arrows show fungal dead cells. The results are representative of two independent experiments.

yeast cells forming microcolonies and three-dimensional structures and few hyphae while biofilms of the *tup1* mutant showed a dense hyphal network (Figs. 6A and 6D). Similar patterns were observed in the presence of K279a *rpfF*, where bacteria grew associated to fungal cells (Fig. 6B and 6E). Once more, mixed cultures of *C. albicans* ATCC 10231 or *tup1* with K279a exhibited biofilms with structurally affected hyphal cells (Figs. 6C and 6F).

The results obtained by the three different approaches show that *S. maltophilia* interferes with the biofilm mode of growth of *C. albicans* and severely affects not only hyphal integrity but also kills yeast cells. DSF could be directly responsible for these effects or may induce virulence gene expression. Bacterial virulence factors might be involved in bacterial-fungal interaction. Hogan and Kolter[13] reported that *P. aeruginosa* forms a dense biofilm on *C. albicans* filaments and kills the fungus, while neither of them binds to nor kills the yeast form of *C. albicans*. Hyphal death occurred only after the onset of biofilm formation, and *P. aeruginosa* virulence factors, including pili and secreted molecules, are important in the killing of *C. albicans* filaments[13]. In *S. maltophilia*, RpfF is essential for the synthesis of DSF, and *rpfF* mutation leads to a coordinate reduction in the synthesis of virulence factors such as extracellular enzymes, biofilm structure and motility[11,31]. *S. maltophilia* formed biofilms on *C. albicans* cells and this contact could favor the effect of virulence factors,

regulated by DSF, in the killing of *C. albicans* filaments but also yeast cells. *S. maltophilia* possess traits associated with biocontrol mechanisms including, among others, extracellular enzymes such as proteases and chitinases[10,18,37]. We have previously mentioned that DSF regulates protease production in this species. StmPr1, the major extracellular protease of clinical strain K279a, has been implicated as a virulence determinant and the environmental strain *S. maltophilia* W81 can protect sugar beet against *Pythium*-mediated damping-off disease through the production of an extracellular protease[7,10].

Minkwitz and Berg[22] characterized 50 *S. maltophilia* isolates from clinical and environmental sources by their *in vitro* antagonism against the human pathogenic fungus *C. albicans* and the plant pathogens *Verticillium dahliae*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. Eight of the 25 clinical isolates (32%) and 21% of the environmental isolates displayed remarkable activity against *C. albicans*. Antifungal activity against plant pathogens was more common among the environmental isolates, although not exclusive to them. All isolates, clinical and environmental, produced proteases and all but two of the environmental isolates produced chitinases.

The genome of K279a (NC_010943) contains the gene *chiA* encoding for chitinase A (Gene ID 6393430), a well-known antifungal trait exhibited by several isolates from the species *S. maltophilia*[7]. A rhizosphere strain of
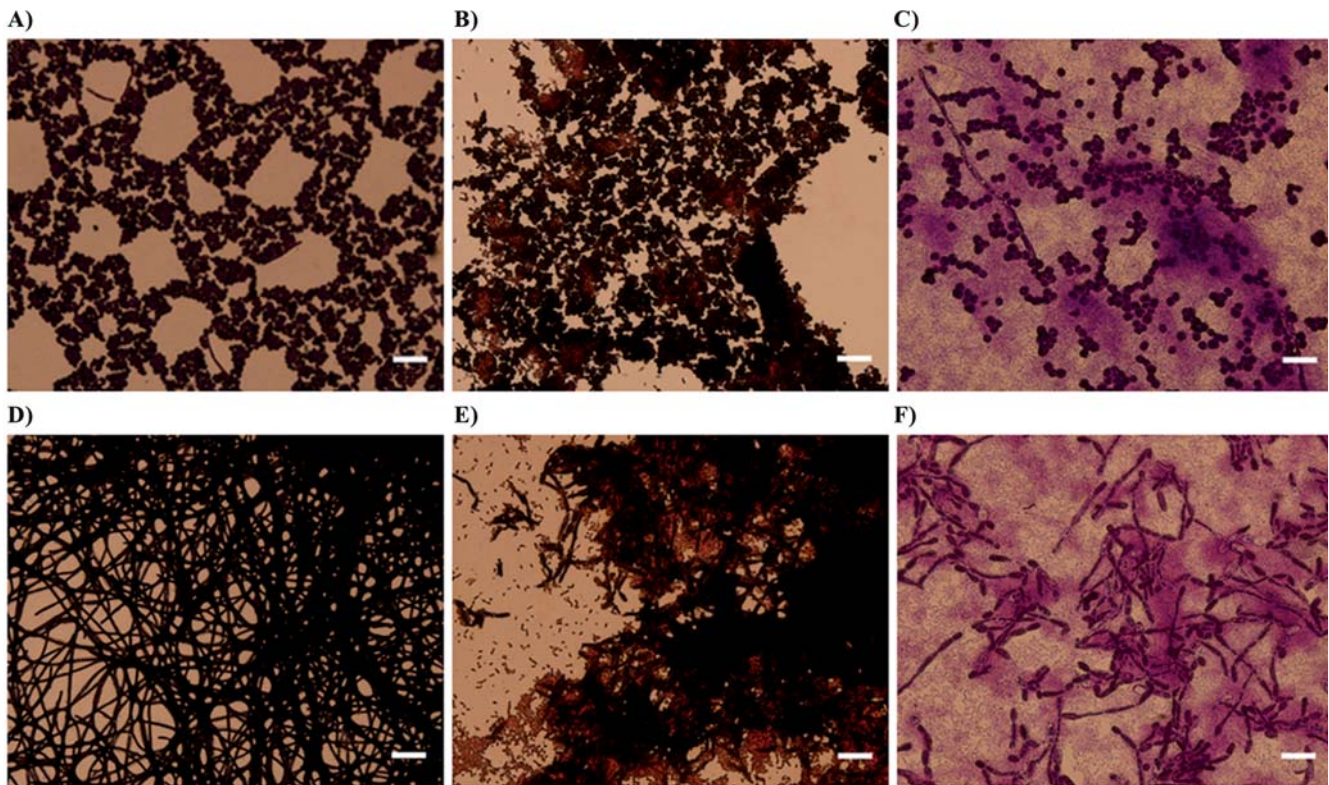
Figure 6 Microscopic analysis of *C. albicans* biofilms in the presence of *S. maltophilia*. *C. albicans* ATCC 10231 (upper panel) and *C. albicans tup1* (lower panel) biofilms were formed on Thermanox cover slips in the absence or presence of bacterial strains. (A) and (D) controls, (B) and (E) cocultures with K279a *rpfF,* and (C) and (F) cocultures with K279a. After 72 hincubation at 37 °C, biofilms were Gram stained and examined by light microscopy (400× magnification). Scale bars correspond to 10 μm.

S. maltophilia, MUJ, is strongly antagonistic towards fungal phytopathogens. Its purified chitinase inhibited the growth of fungi belonging to the genera *Fusarium*, *Rhizoctonia* and *Alternaria*[18]. Furthermore, *S. maltophilia* strain C3, a biocontrol agent of *Bipolaris sorokiniana* in turfgrass, produces at least two chitinases that are antifungal[37]. In *S. maltophilia* there are no reports about DSF regulation of *chiA.* However, in *Xylella fastidiosa* RpfF, which is phylogenetically closely related to the above organism, expression of *chiA* in the *rpfF* mutant is only 40% of that of the wild type[20] and also synthesizes the cell-cell signaling molecule DSF.

The ability of *C. albicans* to switch its morphology between yeast and hyphal form is crucial to its ability to adhere to surfaces[32]. It has been reported that BDSF was more effective against *C. albicans* biofilm formation than farnesol and synthesized DSF, and that BDSF could block *C. albicans* biofilm formation by interfering with the morphological switch[3,36]. Since other members of the DSF family of signal molecules have been implicated in the interkingdom signaling between bacteria and *C. albicans*, it was suggested that DSF from *S. maltophilia* could also influence the yeast-hyphal transition[30,33]. As it was demonstrated in the present study, *S. maltophilia* K279a does interfere with *C. albicans* filamentation and the effect was dependent on DSF production. This interference could also contribute to the antagonistic effect of *S. maltophilia* on *C. albicans* biofilms. Finally, fatty acid signals have been

shown to regulate a wide range of bacterial behaviors including biofilm dispersion. The QS signal *cis*-2-decenoic from *P. aeruginosa* is a member of the DSF family that is able to disperse *C. albicans* biofilms[8]. Thus, further studies are needed to determine whether the antagonistic effects of *S. maltophilia* on *C. albicans* biofilm mode of growth are on adhesion, killing of fungal sessile cells, or on biofilm dispersal.

In conclusion, this is the first study to report that *S. maltophilia* interferes with two key virulence factors of *C. albicans*, the yeast-to-hyphal transition and biofilm formation. DSF could be directly responsible for these effects or may induce the expression of genes involved in antifungal activity. A previous work has established the regulatory influence of DSF from *S. maltophilia* as an interspecies signal. *P. aeruginosa* can respond to DSF by altering its biofilm architecture and increasing tolerance to the antibiotic polymixin[31]. Further studies are needed to determine the potential role of QS and DSF signal under *in vivo* conditions, for example, in the lungs of CF patients where *S. maltophilia* may coexist with *C. albicans*[34].

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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