Appl. Environ. Microbiol. doi:10.1128/AEM.04070-15

Analysis of mechanisms of bacterial (*Serratia marcescens*) attachment, migration and killing of fungal hyphae.

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Running Title: Bacterial migration and killing of fungal hyphae

Keywords: *Zygomycota*, *Mucorales,* *Serratia marcescens,* fungal-bacterial interaction, migration, adhesion.

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Abstract

We have found a remarkable capacity of the ubiquitous gram negative rod bacterium *Serratia marcescens* to migrate along and kill the mycelium of zygomycete molds. This migration was restricted to zygomycete molds, and several *Basidiomycete spp.* No migration was seen on any mold of the phylum *Ascomycota*. *S. marcescens* migration did not require fungal viability or surrounding growth medium, as bacteria migrated along aerial hyphae as well. *S. marcescens* did not exhibit growth tropism towards zygomycete mycelium. Bacterial migration along hyphae proceeded only when the hyphae grew into the bacterial colony. *S. marcescens* cells initially migrated along the hyphae, forming attached microcolonies that grew and coalesced to generate a biofilm covering and killing the mycelium. Flagellum-defective strains of *S. marcescens* were able to migrate along zygomycete hyphae, although significantly slower than the wild-type strain and were delayed in fungal killing. Bacterial attachment to the mycelium does not necessitate type 1 fimbrial adhesion since mutants defective in this adhesin migrated equally well or faster than the wild- type strain. Killing does not depend on the secretion of *S. marcescens* chitinases as mutants in which all three chitinase genes were deleted retained wild-type killing abilities. Better understanding of the mechanisms by which *S. marcescens* binds, spreads and kills fungal hyphae could serve as an excellent model system for such interactions in general; fungal killing could be employed in agricultural fungal biocontrol.

Introduction

Prokaryote - eukaryote interaction are ubiquitous. Although the relationships between bacteria and plants or animals have gained the most attention, encounters among bacteria and fungi are likely far more common. Several studies have demonstrated numerous possible interactions between bacteria and fungi, including mutualism, commensalism, and pathogenesis (1-4). Hogan et al. (5) showed that the pathogenic gram negative bacterium *Pseudomonas aeruginosa* is able to attach to, spread on and kill the dimorphic pathogenic fungus *Candida albicans* in its filamentous form. *P. aeruginosa* initially forms discrete hyphal-attached bacterial colonies followed by biofilm formation (6). Spreading is reduced on the yeast form of *C. albicans* (7). In a phenomenon called "bacterial hitchhiking" *Staphylococcus aureus* is able to invade host tissue and disseminate via adherence to the invasive hyphal elements of *C. albicans* (8). Furuno et al. (1) showed that dispersal of the bacterium *Pseudomonas putida* across air gaps, simulating non-saturated soil, was made possible along the mycelium of the plant pathogenic fungus *Pythium ultimum*, suggesting a mechanism for soil bacterial translocation across soil air-pockets.

Preliminary research in our laboratory found that a non-pigmented strain of *S. marcescens*, isolated from compost, was capable of rapidly traveling over the mycelium of the fungus *Rhizopus oryzae* and other species of fungi from the phylum *Zygomycota*. To date and to the best of our knowledge, a trophic interaction between the bacterium *S. marcescens* and zygomycete fungi has not been described.

Serratia spp. are ubiquitous inhabitants of soil, water and plant surfaces, and are commonly associated with food spoilage. *S. marcescens*, in particular, represents an important nosocomial pathogen capable of causing pneumonia, intravenous catheter-

associated infections, ocular and urinary tract infections, osteomyelitis and endocarditis (9).

S. marcescens is unique among enteric bacteria in many aspects. It secretes extracellular DNase, gelatinase, lipase, several proteases, a red pigment (prodigiosin), three chitinases and a chitin binding protein. It is in fact believed to be one of the most efficient chitin degrading bacteria in the environment (10).

Fungi of the order *Mucorales*, phylum *Zygomycota*, are filamentous, aseptate molds. Most members of the *Mucorales* are thermotolerant saprotrophs, and are common worldwide in soil and in decaying matter. Mucormycosis, an infection caused by fungi of the order *Mucorales*, is an uncommon, yet often deadly infection in immunocompromised patients (11). It is noteworthy that zygomycetes are the only members of the fungal kingdom whose cell wall is primarily composed of chitin (12) that is susceptible to enzymatic breakdown by chitinases secreted by *S. marcescens* (10).

To define the complex interactions between the bacterium *S. marcescens* and fungi, we tested the hypothesis that the bacterium has the ability to use fungal hyphae as a mean of translocation, and that this ability is most significant when in contact with fungi of the phylum *Zygomycota*. We also attempted to identify the molecular mechanisms underlying the ability of *S. marcescens* to spread along and kill fungal mycelia. It has been suggested in the past that some of the bacterial virulence arsenal used against humans has evolved to combat microbial eukaryotes found in their surroundings, therefore a better understanding of these microscopic encounters may help to improve both bacterial infection prophylaxis and fungicidal therapies.

Materials and Methods

Organisms and culture conditions. All fungal strains used in this study are listed in Table 1. Fungi were cultivated on YAG (0.5% [wt/vol] yeast extract, 1% [wt/vol] glucose, 10 mM $MgSO_4$, 1.5% [wt/vol] agar), supplemented with trace elements and vitamins. Spores were harvested in 0.2% (vol/vol) Tween 20, resuspended in double-distilled water, and counted with a hemocytometer. Due to lack of spore formation, basidiomycete strains were cultured by seeding mycelial plugs, ~2 mm^2 in size, from a 2-5 day old mycelium grown on YAG at 37˚C. Mycelia were harvested with a sterile surgical blade under aseptic conditions.

Bacterial strains used in this study are listed in Table 1. Unless otherwise specified, all strains were cultivated at 37˚C on modified SOC (0.5% [wt/vol] yeast extract, 2% [wt/vol] tryptone, 0.5% [wt/vol] glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 1.5% [wt/vol] agar), in the presence of antibiotic selection as required for each strain. Optical density (OD) was determined spectrophotometrically at 600 nm. Antibiotics used in the study were ampicillin (100 µg/ml), chloramphenicol (20 µg/ml), gentamycin (10 µg/ml) and kanamycin (100 µg/ml).

Qualitative measurement of *S. marcescens* spreading over fungal mycelium. *Ascomycota/Zygomycota* and *S. marcescens* 1 or RM66262 were inoculated as streaks at a 45 degree angle on solid medium and incubated at 30˚ or 37˚C for 48 h. Inoculation was made by streaking spore/bacterial suspension; the first streak was allowed to dry thoroughly prior the next, to avoid dragging the spores along the path of the bacteria. Bacterial spreading along the mycelium was assessed visually.

For non-sporulating basidiomycetes, mature mycelial plugs were seeded on a SOC plate, forming a line. After 2-5 days at 30˚C, 10 µl of *S. marcescens* 1 or *S.*

marcescens RM 66262 suspension (O.D = 0.5) were inoculated adjacent to the newly formed mycelium, and incubated at 30˚ or 37˚C. Bacterial spreading along the mycelium was visually evaluated.

Quantitative measurement of *S. marcescens* spreading over *R. oryzae 3465* mycelium. To measure the rate of bacterial spreading over fungal mycelia, fresh spores of *R. oryzae 3465* were spread over a SOC plate at a concentration of 2.5 x 10^4 spores/cm^2, allowed to germinate for 12 h at 37˚C, then inoculated at the center of the plate by pipetting 5 µl bacterial suspension grown at 30˚C in SOC to exponential phase (OD=0.5), on the agar surface. After incubation at 30˚C, the plates were sampled by insertion of a 96 pin metal replicator (of which 66 pins fit into the plate) into the agar and replication onto fresh SOC plates at 4, 8, 12 and 24 h post inoculation. Replicated plates were incubated for 24 h at 37˚C, when bacterial colonies were clearly visible. The number of positive pinned colonies was used as an estimate for the rate of bacterial spreading. Student's *t*-test was used to determine the statistical significance of bacterial spreading at each time-point. Significance was defined at a *P* value of 0.05.

***S. marcescens* killing of *R. oryzae 3465* mycelium.** Fresh spores of *R. oryzae 3465* were spread over 24-well SOC agar plates (1.5 ml SOC/well) at a concentration of 2.5 x10^4 spores/well and allowed to grow for 12 h at 37˚C. Each mycelium-covered well was then inoculated at the center by pipetting 5 µl of *S. marcescens* suspension grown at 30˚C in SOC to exponential phase (OD = 0.5). Plates were incubated at 30˚C for the indicated time periods. At each time-point the agar plug (containing bacteria and mold) was removed from the well with a sterile spatula, placed inverted on the surface

of a SOC agar plate containing antibiotics for 24 h at 37˚C and scored for radial growth of the fungus. No detectable fungal growth was defined as 100% killing by the bacteria.

***S. marcescens* migration over dead fungal mycelium.** 10^5 *Rhizopus oryzae* spores were grown in a petri dish containing 20 ml liquid YAG at 37˚C for 24 h, forming a thick mycelium in which sporulation had not begun. The medium was then gently suctioned from the plate and the mycelium frozen intact at -70˚C and lyophilized. For preparation of *A. fumigatus* mycelium, 10^5 *A. fumigatus* spores were grown in 100 ml liquid YAG under shaking at 37˚C for 24 h. The mycelial suspension was poured over miracloth to form a thin layer, dried, frozen intact at -70˚C and lyophilized.

Mycelia were then exposed to UV radiation (wavelength 312 nm, 10,000 µW/Cm^2, 60 min each side of the mycelium) on a Vilber lourmat ECX-F15m UV table. Complete mycelial death was confirmed by placing them on solid SOC agar for 24 h during which no growth was seen. Bacterial migration was tested by cutting the dead mycelium into triangles with a sterile surgical blade, placing the pieces 2-3 mm apart from a bacterial colony made of 5 µl logarithmic phase (OD = 0.5) bacteria suspended in SOC. Mycelium and bacterial colony were placed on the plate simultaneously but without contact to avoid capillary suction of bacteria along the hyphae, instead, the bacterial colony was allowed to grow for 24-48 h at 30˚C or 37˚C until contact was naturally made with the edge of the mycelium.

***S. marcescens* movement over aerial fungal hyphae.** The experiment was conducted on plastic stages raised ~3 mm inside a petri dish and separated by ~3 mm. The plastic stages were bent to the desired shape, autoclaved, and aseptically fixed to

a sterile, dry petri dish with epoxy glue. Humidity was maintained by pouring 3 ml sterile 1.5% [wt/vol] agar solution on each side of the plate. Blocks of SOC agar were cut off a freshly poured plate and placed on top of the stages, inoculated with *Rhizopus oryzae* spores and *S. marcescens 1* suspension at opposite sides and incubated at 37˚C for 48-96 h. to allow bridging of the gap by the fungal hyphae. Bacterial movement was confirmed by microscopy and by sampling the fungal mycelium with a sterile wooden stick, these samples were point-inoculated on solid SOC agar and grown for 24 h at 37˚C.

Fluorescence microscopy of *S. marcescens* attachment to fungal hyphae on solid medium. *R. oryzae 3465* spores were placed and allowed to germinate at 37˚C overnight on SOC agar plates. 5 µl suspension of *S. marcescens* RM66262 pGFP grown in liquid SOC (OD = 0.5) was placed as a droplet next to the fungal hyphae. The plates were incubated overnight at 37˚C and viewed by fluorescence microscopy on an Olympus IX50 microscope equipped for fluorescence, at a magnification of ×400. Images were recorded with a digital Olympus DP70 camera.

Scanning electron microscopy (SEM) analysis. *S. marcescens* RM66262 and *R. oryzae 3465* were grown on SOC agar plates as described in the previous section. Fixation and processing of samples for SEM was performed essentially as described (13). Briefly: 5 x 5 mm fragments were carefully excised from the zone of interaction and vapor fixed with 8% paraformaldehyde and 4% glutaraldehyde dissolved in water for 1 h in a closed chamber. Secondary vapor fixation was then performed in an aqueous solution of 2% osmium tetroxide for 1 h. The samples were submerged for 10 min in DDW and then dehydrated (10 min, twice for each step) under a series of

ethanol concentrations (7.5%, 15%, 30%, 50%, 70%, 90%, 95%, and 100%). Next, samples underwent critical point drying (CPD) using a K850 CPD dryer (Quorum Technologies, UK). Coating was performed with 3 nm iridium using a Q150T coater (Quorum Technologies, UK). Samples were imaged with a Merlin scanning electron microscope (Zeiss, Germany).

Results

***S. marcescens* migrates along zygomycete and some basidiomycete molds.** While studying the interaction between various bacteria and molds isolated from compost heaps, we noticed that a bacterial *S. marcescens* isolate was able to spread rapidly over the mycelium of zygomycete molds isolated from the same microenvironment, whereas no spreading of *S. marcescens* over *A. flavus* mycelium or the surrounding agar surface was seen (Fig. 1). To better define this phenomenon, 26 fungal spp. representing three phyla (Table 1), were tested against one red-pigmented environmental strain of *S. marcescens* 1 and one non-pigmented patient isolated strain (RM66262*)* at 30˚ or 37˚ C on 1.5% SOC agar. We decided to use two temperatures since Lai et al. (14) showed that a wild-type clinical isolate of *S. marcescens (*CH-1*),* exhibited a characteristic swarmer phenotype on L-broth solidified with 0.8% agar at 30˚C, but no swarming was noted on identical plates incubated at 37˚C even after 48 h. Results were assessed visually following photography of the plates. Both strains of *Serratia* migrated along all zygomycete species tested at both 30˚ and 37˚C whereas no migration was seen along ascomycete fungi (*Aspergillus* and *Penicillium*). Interestingly, both *S. marcescens* 1 and *S. marcescens* RM66262 migrated along three basidiomycete species: *Phanerochaete chrysosporium*, *Ustilago sphaerogena* and *Gleophyllum trabeum.* Both strains migrated over the mycelial periphery of *Lentinus lepideus* and *Ganoderma lucidum* and did not migrate along the mycelia of the basidiomycetes *Alternaria alternata, Cladosporium cladosporoides, Pleurotus ostreatus, Pleurotus pulmonarius, Schizophyllum commune* and *Trametes versicolor* (Table 1).

S. marcescens does not actively chemotax toward _R. oryzae_. We hypothesized that under optimal conditions for bacterial migration, *S. marcescens* may exhibit active chemotaxis toward the fungal colony. To test this, we point-inoculated *S. marcescens* RM66262, next to point inoculated *R. oryzae* 3465 on SOC agar. Following incubation of 24 h at 37˚C, no change was seen in the shape or outline of the bacterial colony towards the fungal colony (Fig. 2A). Interestingly, fungal growth was reduced in the vicinity of the bacterial colony. Bacterial migration commenced only after hyphae made contact with the edge of the bacterial colony, suggesting the necessity for physical contact (Fig. 2B). Similar results were seen with either *S. marcescens 1* or RM66262 point inoculated next to *R. oryzae 3465* at 30˚C (not shown).

S. marcescens migrates over dead fungal hyphae. To test the ability of *S. marcescens* to migrate over killed fungal hyphae, *S. marcescens* 1 was inoculated 2-3 mm from the edge of UV-killed and lyophilized *R. oryzae 3465* or *A. fumigatus* mycelium. As controls we used live mycelia. As shown above, live *R. oryzae* mycelium supported bacterial migration, whereas live *A. fumigatus* mycelium did not. Interestingly, *S. marcescens* 1 in contact with live *A. fumigatus* showed decreased color (prodigiosin production) possibly as a result of toxins produced by the fungus. Dead *R. oryzae* and *A. fumigatus* mycelia were both entirely covered by bacteria after 36 h incubation at 30˚C (Fig. 3). Expansion in other directions, or beyond the margin of the mycelium, was negligible. Similar results were seen at 37˚C (not shown). These findings suggest that (i) Live *A. fumigatus* actively repels invasion by *S. marcescens*, whereas *R. oryzae* does not. (ii) Dead *A. fumigatus* does not actively repel *S. marcescens*, allowing it, like *R. oryzae*, to serve as a passive scaffold and highway for the spread of the bacteria.

S. marcescens can migrate across aerial hyphae directly without contacting the agar surface. To test the ability of *S. marcescens* to migrate exclusively along fungal hyphae we designed a model containing two separate blocks of agar, one inoculated with *S. marcescens* 1, the other with *R. oryzae 3465* or *R. oryzae 5698* separated by an aerial gap (Fig 4A-C). Movement between the isolated blocks is only possible via the bridge of aerial hyphae formed between them after ~48 h of incubation at 37ºC in a humid chamber. Successful bacterial "bridging" was defined by positive growth of the bacterium on the fungal agar block or the fungal mycelia bridging the air gap after sampling with a wooden toothpick. After 96 h of incubation, bacteria were found on hyphae bridging the air gap in 100% of the *R. oryzae* samples. In half of the samples, bacteria were found on the opposing agar block inoculated with *R. oryzae* alone, indicating that they had both bridged the gap and colonized the fungus (Table 2). Microscopic examination clearly identified red patches of bacteria migrating over the bridging aerial hyphae (Fig. 4D).

Microscopic analysis of _S. marcescens_ movement along _R. oryzae_ hyphae on solid medium by fluorescence and SEM microscopy. For fluorescence microscopy, *R. oryzae 3465* mycelia and adjacently inoculated *S. marcescens* RM66262-pGFP were grown on SOC agar until physical contact between the organisms occurred. The interaction was followed by light and fluorescent microscopy. *S. marcescens* migration began ~60 min after hyphae penetrated the bacterial colony. Interestingly, most bacteria could be seen moving and forming small microcolonies along the hyphae after 90 min, while some moved along the agar surface between hyphae (Fig. 5, 90'). Bacterial biofilm formation occurred after ~4 h as the microcolonies expanded

and coalesced, forming a thick layer around the hyphae (Fig. 5, 4 h time-point). High resolution surface imaging by SEM reinforced these observations, highlighting the aggregation of microcolonies along the hyphae during early migration and (Fig. 6 A-D), and the subsequent formation of a mature biofilm covering the mycelium (Fig. 6 E-F).

S. marcescens mutants lacking a flagellum show impaired migration and killing of _R. oryzae 3465_ mycelia. Flagella have been shown to be important for many types of active bacterial translocation. We therefore hypothesized that flagellar expression is important for *S. marcescens* migration over fungal mycelia. To test this, motility of *S. marcescens* RM66262 strains *ΔflhD* (lacking $FlhD_2C_2$, the flagellar master regulator operon) and *ΔfliA* (lacking FliA, a transcriptional factor specifically required for the control of the final stages of flagellar assembly, downstream of $FlhD_2C_2$) was compared to the *S. marcescens* RM66262 isogenic wild -type. Spreading motility was measured on a mycelial-covered plate (*R. oryzae 3465)* as described in Materials and Methods. Our results show that spreading of the *ΔfliA* and *ΔflhD* mutants was slower than the wild-type (Fig. 7A). Interestingly, the killing ability of the *S. marcescens ΔfliA* and *ΔflhD* mutants was diminished (Fig. 7B). Whereas the wild-type *S. marcescens* strain killed *R. oryzae 3465* after 36 h of co-inoculation, the mutant strains took 48 h. This is not due to their impaired spreading: the killing assay measures the time needed to kill a mycelium growing on a very small 1.5 cm agar plug that is completely covered by *S. marcescens* in ~ 4 h (see Material and Methods).

Because the expression of the *Serratia* phospholipase PhlA is transcriptionally regulated by FliA and its secretion depends on the integrity of the flagellar secretome (15), we tested whether PhlA could be responsible for the reduced killing observed for

the *ΔflhDC* and *ΔfliA* mutant strains. Moreover, because the levels of expression of the pore-forming toxin ShlA (coded by the *shlBA* operon) were previously shown to be controlled by the FlhDC-FliA regulatory cascade (16, 17), we also assayed the *ΔshlB* mutant strain for the *R. oryzae 3465* killing capacity. However, there were no differences in the killing values of the *ΔphlA* or the *ΔshlB* mutant strains when compared to the wild-type strain (data not shown) indicating that the altered expression of these secreted enzymes in the *ΔflhD* or *ΔfliA* mutant strains is not responsible for the observed attenuated killing phenotype although we cannot rule out that deletion of both *ΔphlA* and *ΔshlB* together may contribute synergistically to fungal killing.

***S. marcescens* defective in fimbrial expression migrate faster along *R. oryzae* 3465 mycelia**. Type 1 fimbria play a significant role in bacterial attachment to biotic and abiotic surfaces. To determine whether attachment by type 1 fimbriae affects bacterial migration over fungal mycelia, we compared the migration rates of *S. marcescens* CMS 515 (*oxyR2*, a fimbria-lacking mutant lacking the OxyR transcription factor that activates the *fimABCD* operon) and CMS 629 (*fimC* mutant usher gene required for fimbria formation) to CMS 376 wild-type and CMS 519 (the *oxyR2* mutant revertant which generates fimbria like the wild type) (18). These results show that bacteria defective in fimbrial expression migrate faster along the mycelia of *R. oryzae 3465* (Fig. 8A). These results suggest that fimbrial adhesion impedes migration, perhaps through the formation of a dense bacterial biofilm or tighter adhesion to the hyphal mycelium. Loss of fimbria in the mutant strains did not affect their ability to kill *R. oryzae 3465* mycelia at the same rate as the wild-type strain (Fig. 8B), suggesting that killing does not necessitate fimbria-mediated attachment.

Secreted *S. marcescens* chitinases are not involved in killing *R. oryzae 3465* mycelia. Because *S. marcescens* produces extraordinary amounts of secreted chitinases and the zygomycetes contain an unusually high proportion of chitin in their cell walls, we hypothesized that fungal killing is mediated by secreted *S. marcescens* chitinases. We therefore compared the spreading and killing activity of a no-Chi (*ΔchiA, ΔchiB, ΔchiC*) triple mutant lacking any detectable secreted chitinase activity to that of the isogenic wild-type Dba10 strain (19). There was no difference in the ability of the no-Chi mutant to spread along *R. oryzae 3465* mycelia relative to the wild-type strain (Fig. 9A) and more surprisingly, the killing ability of the mutant was completely unaffected (Fig. 9B). This suggests that the ability of *S. marcescens* to kill the fungus depends on an as-yet unidentified pathway. Secreted *S. marcescens* chitinases may be involved in digesting the fungus after it is killed.

Discussion.

Previous unpublished work undertaken in our laboratory showed that an environmental strain of *S. marcescens* isolated from a compost heap in Israel rapidly and efficiently migrated along mycelia of zygomycete molds grown on agar plates. Here, we describe a series of experiments to better understand this phenomenon.

Migration of *S. marcescens* over fungi has some specificity. A migration screen between two strains of *S. marcescens* and numerous fungi from different divisions revealed that while the bacterium was able to migrate along the mycelium of all eight species of zygomycetes tested, no migration was observed with seven Ascomycete species, while basidiomycete species gave mixed results, with two out of ten species enabling strong migration while others allowed only peripheral migration or none at all. These results can be explained by two alternative non-exclusive hypotheses (i) there is a species/phylum-specific interaction between particular cell surface bacterial and fungal determinants (i.e. adhesins), that mediates attachment and movement. Bacterial attachment and movement occurs only on fungi possessing these determinants. (ii) Some fungal species/phyla produce surface or secreted defense factors that block bacterial interactions. Bacterial attachment and movement occurs only in fungi lacking these defenses. We showed that dead *A. fumigatus* mycelium supports bacterial migration while live mycelium repels it. This suggests that the live mycelium secretes factors that block bacterial interaction and migration, supporting the second hypothesis at least for *A. fumigatus*. The identity of these bacterial-repelling fungal factors is of considerable interest and will be investigated in our future work.

S. *marcescens* mycelial migration is an active, ordered process. *S. marcescens* was able to migrate along architecturally preserved UV-killed *R. oryzae* mycelia, suggesting that bacterial translocation is an active bacterial-dependent process that is not influenced by hyphal growth and transport. Migration also occurred along fungal hyphae suspended in the air, suggesting that the bacteria possess a means of attachment to the fungal cell wall surface. *S. marcescens* hyphal migration appears to follow three steps; (i) initial rapid movement of individual bacteria along or between the fungal hyphae (ii) attachment of these "colonizer" bacteria and the formation of discrete bacterial colonies and finally (iii) the coalescing of these colonies to form a biofilm.

Flagellar expression supports *S. marcescens* migration over fungal hyphae, but is not the only mode of transport. The hypothesis that bacterial translocation along hyphae was flagellum-dependent was tested using *S. marcescens* Δ*flhD* (encoding the flagellar expression master operon) and Δ*fliA* (encoding an alternative sigma factor, expressed during flagellar assembly) mutant strains (20). Both mutants showed greatly decreased swarming and swimming in standard tests (data not shown). Both the Δ*flhD* and Δ*fliA* mutations strongly reduced the rate of bacterial expansion over *R. oryzae* mycelium, but they both nevertheless spread over approximately half of the entire agar surface of the plate after 24 h of co-incubation. These results might be explained by the existence of an additional non-flagellar dependent mode of transportation that enables slower movement along hyphae such as "sliding" mediated by quorum sensing and the production of surface tension modifying surfactants. Surfactant production and flagellar expression are regulated by different genes and environmental signals. The sliding hypothesis can be tested with mutant strains

defective in surfactant production (21). Interestingly, *Pseudomonas putida* bacteria lacking flagella lose their ability to migrate along hyphae suggesting that unlike *S. marcescens,* they rely entirely on flagella to move along the fungal mycelium (22).

Our finding that killing of *R. oryzae* mycelium was delayed in the Δ*flhD* and Δ*fliA S. marcescens* strains could be due to the fact that the flagellum also functions as a secretion system with structural and functional relatedness to the type-three secretion system. The flagellum is able to translocate not only the components involved in self-assembly but also for the secretion of non-flagellar proteins (23). Non-flagellar effectors secreted by the flagellum in *S. marcescens* include PhlA phospholipase (15). Furthermore, ShlA expression has been demonstrated to be activated by FliA at the transcriptional level (16). However, in a preliminary analysis we found that *S. marcescens phlA* and *shlA* null mutants retained wild-type fungal killing abilities. Nevertheless, we cannot rule out that other yet unknown protein/s that could be transcriptionally dependent on the flagellar regulatory cascade or secreted by the flagellar secretome exert part of the observed fungal mycelium killing by *Serratia*.

S. marcescens attachment to fungi and the role of type 1 fimbriae. We reasoned that *S. marcescens* mutants lacking type 1 fimbriae, hair-like appendages that enable the bacterium to attach to biotic and abiotic surfaces, will have a reduced capacity for migration over fungi. Two fimbria-defective mutants (*oxyR* and *fimC*) spread faster during the first 8- 12 hours. Thus, we propose that the attachment of the afimbrial mutants to hyphae is weaker, enabling them to undergo faster initial migration. Another possible explanation is the intimate involvement of fimbriae in bacterium-bacterium attachment and biofilm formation; reduced inter-bacterial attachment may "free" the colony for faster movement and expansion.

S. *marcescens* fungal killing does not depend on the secretion of chitinases. *S. marcescens* secretes unusually high levels of three extracellular chitinases (*ChiA, ChiB*, and *ChiC*) enabling it to use chitin as a source of nitrogen and carbon (10, 19). We hypothesized that *S. marcescens* utilizes secreted chitinases to specifically target and kill zygomycete molds because they contain unusually high levels of chitin (in the form of chitosan) in their cell walls (12). Surprisingly we found that a *S. marcescens* triple deletion mutant (*ΔchiA/ΔchiB/ΔchiC*) with no detectable secreted chitinase activity retained wild-type fungal killing abilities. We propose that *S. marcescens*-mediated fungal killing is carried out by additional as-yet-unknown virulence factors and that chitinases are used to degrade the cell wall during the next step of digestion and assimilation. *P. aeruginosa* killing of *C. albicans* hyphae is understood in much greater detail-it involves both contact mediated and soluble secreted factors such as phospholipase C and phenazine derivatives (5, 6, 24) suggesting similar approaches to pursue in our future studies.

It is interesting to compare the *S. marcescens-Zygomycota* interactions we have described here to those found for *P. aeruginosa-C. albicans* interactions, which are the most extensively studied system of bacterial-fungal interaction. In both cases and over similar time-scales the bacteria formed biofilms on the hyphal surface leading to fungal death (5). However, *P. aeruginosa-C. albicans* binding occurs in liquid medium, and unlike *S. marcescens, P. aeruginosa* does not rapidly spread over fungal mycelia growing on agar surfaces (5). While flagellar defective *S. marcescens* were delayed in spreading and killing of the fungus, similar *P. aeruginosa* mutants showed normal killing. While pili-deficient *S. marcescens* showed increased spreading rates along hyphae and normal killing, *P. aeruginosa* mutants showed normal biofilm

formation but delayed killing. These findings suggest that the molecular mechanism of *S. marcescens-Zygomycota* interaction differs substantially from that of *P. aeruginosa* and *C. albicans.*

In summary, our characterization of the mechanisms allowing *S. marcescens* to migrate over fungal mycelia revealed a complex, multi genetic, multifactorial system. The complexity of the interaction, taking place on solid agar surfaces and not in suspension in liquid medium make it difficult to analyze experimentally. It is unclear whether the fungi serve as a highway, a nutrient source or both. The chemical and physical nature of bacterial attachment seems to involve numerous mechanisms, and the common properties of the fungi found to support or repel migration will have to be further characterized as well.

Acknowledgments: we would like to thank Professor Frank Sargent from the University of Dundee UK for their generous donation of bacterial mutant strains; Dana Levinson from the faculty of Agriculture, Food and Environment, Rehovot, Israel for the generous donation of fungal basidiomycete strains.

Funding: This study was supported by the Binational Science Foundation (BSF) 2011322 grant to N.O

Transparency Declaration: None to declare

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Figure legends

Fig. 1. *S. marcescens* spreads over *Rhizopus oryzae* mold. *S. marcescens 1* (red) was co-cultured at a 45 degree angle to various mold species (white) (A) *Aspergillus flavus Afl1* and *S. marcescens* 1 after 48 h of co- incubation. No spreading of *S. marcescens* over *A. flavus* mycelium is seen. (B, C, D) *R. oryzae* and *S. marcescens* 1, after 24, 30 and 48 h incubation, respectively. Spreading of pigmented *S. marcescens* over *R. oryzae* mycelium can be clearly seen preceding the growing hyphal tips at 24 and 30 h (arrows) and covering the entire mycelium after 48 h. All samples were incubated at 37˚C on SOC plates.

Fig 2. *S. marcescens* strain RM66262 does not chemotax towards *R. oryzae 5698*. Bacterial and fungal strains were point inoculated adjacently. No visible change in bacterial colony morphology is observed, indicating there is no chemotaxis (A). *S. marcescens* migration begins only after hyphae have invaded the bacterial colony (B). Isolates were grown on SOC at 37˚C for 24 h. Identical results were seen with *S. marcescens* 1 and *R. oryzae 3465.*

Fig. 3. *S. marcescens 1* migrates over killed mycelium of *R. oryzae 3465* and *A. fumigatus*, but fails to migrate on live *A. fumigatus* mycelium. Following 36 h of incubation at 30ºC degrees on SOC plates, red bacterial coloration is visible on the entire mycelial surface and edges of live or killed *R. oryzae* mycelium (top panel). In contrast, *S. marcescens* spreads on killed *A. fumigatus* mycelium but fails to do so on live mycelium (bottom panel).

Fig. 4. *S. marcescens 1* crosses an aerial bridge of *R. oryzae 3465* hyphae. (A-B) Scheme of bridging experiment plate, containing two separate raised SOC agar blocks (red in B) inoculated with bacterial and fungal inoculum separately and incubated in a humid chamber. To eliminate bacterial migration in the water condensate, the SOC agar blocks are placed over raised plastic stages (yellow in B and C). Humidity is maintained by pouring 3 ml 1.5% agar solution at each side of the plate (colorless in B). (C) The air gap is bridged by fungal hyphae after less than 48 h at 37˚C. Box enlarged in (D) shows *S. marcescens* 1 (red arrow) spreads along *R. oryzae 3465* aerial hyphae (red arrow) bridging the gap between the agar blocks after 48 h incubation at 37˚C. (Top view with binocular microscope).

Fig. 5. Fluorescence microscopy of *S. marcescens* interacting with *R. oryzae* mycelium. *S. marcescens* 66262/pGFP and *R. oryzae 3465* were inoculated adjacently on SOC agar plates and incubated at 37˚C until the fungal hyphae came into physical contact with the bacterial colony (t = 0'). Light and fluorescent (GFP) microscopy performed on the contact-region at different time-points. Scale bar = 50 µm.

Fig. 6 High-resolution SEM imaging of *S. marcescens* interacting with *R. oryzae* mycelium. *S. marcescens* RM66262 forms discrete colonies on *R. oryzae 3465* hyphae during early (1-2 h) contact on the surface of SOC agar plates (A, enlarged inset B), on aerial hyphae (C, enlarged inset D), later (4h) forming a mature biofilm (E, enlarged inset F) covering the hyphae. Note numerous bacteria visible just below the surface of the biofilm (E) that can be clearly seen where the biofilm surface is disrupted (F).

Fig. 7. *S. marcescens* mutants lacking a flagellum show impaired migration and killing of *R. oryzae 3465*. (A) To measure bacterial spreading, *S. marcescens* RM66262 WT, *ΔflhD* (defective in flagellar master regulator) and *ΔfliA* (defective in flagellar class 2 gene) were inoculated separately in the center of SOC agar petri dishes coated with 12 h old *R. oryzae 3465* mycelia, and incubated at 30˚C. Bacterial migration was measured by periodic replication of the plates onto fresh SOC medium. The number of new bacterial colonies was used as a marker of bacterial migration rate. (B) To measure hyphal killing, *S. marcescens* strains were inoculated in the center of SOC 1.5 cm wells coated with 12 h old *R. oryzae 3465* mycelia, and incubated at 30˚C. Hyphal killing was measured by removing the agar plugs from the wells, incubating them on SOC-kanamycin plates and scoring for fungal growth as described in the Materials and Methods. Figure is a representative of three experiments showing an average ± SD of triplicates. ** $P < 0.005$ between mutant and WT strain.

Fig. 8. **Fimbria-defective *S. marcescens* mutants migrate faster along *R. oryzae* hyphae**. (A) Migration along preformed *R. oryzae 3465* mycelium by *S. marcescens CMS376* WT, *ΔoxyR2* mutant (defective in oxidative stress reaction and fimbriae expression) and *ΔfimC* (defective in fimbriae structural genes). Strains were inoculated separately on the center of *R. oryzae 3465* mycelium, and incubated at 30˚C. Bacterial migration was measured as described in Materials and Methods. (B) Killing of hyphae by *S. marcescens* fimbria-defective mutants is unchanged relative to that of the *CMS376* WT strain. Figure is a representative of three experiments

showing an average ± SD of triplicates. *P < 0. 05, ** P < 0.005, *** P < 0.0005, between mutant and WT strain.

Fig. 9. *S. marcescens* migration and killing of *R. oryzae 3465* does not depend on its ability to secrete chitinases. (A) To measure bacterial spreading, *S. marcescens* Db10 WT, and Db10-NoChi strain (lacking detectable secreted chitinase activity) were inoculated separately in the center of SOC agar petri dishes coated with 12 h old *R. oryzae 3465* mycelia, and incubated at 30˚C. Bacterial migration was measured by periodic replication of the plates onto fresh SOC medium. The number of new bacterial colonies was used as a marker of bacterial migration rate. (B) To measure hyphal killing, *S. marcescens* strains were inoculated in the center of SOC 1.5 cm wells coated with 12 h old *R. oryzae 3465* mycelia, and incubated at 30˚C. Hyphal killing was measured by removing the agar plugs from the wells, incubating them on SOC-kanamycin plates and scoring for fungal growth as described in the Materials and Methods. Figure is a representative of three experiments showing an average ± SD of triplicates.

Table 1. Bacterial and Fungal Strains used in this study

Species/strain	Comments
S. marcescens 1	Environmental isolate, pigmented
S. marcescens RM66262, wild-type	clinical isolate non-pigmented (25)
S. marcescens 66262/GFP	pBBR1-MCS2:promLac-GFP (20)
S. marcescens 66262/Δ*flhD*	RM66262 *flhD*::pKNOCK-Cm (20)
S. marcescens 66262/Δ*fliA*	RM66262 *fliA*::pKNOCK-Cm (16)
S. marcescens 66262/Δ*shlB*	shlB::pKNOCK-Sp (16)
S. marcescens 66262/Δ*phlAB*	Δ*phlAB*::Cm (26)
S. marcescens CMS376, wild-type	Originally from Presque isle culture, strain number 3611, pigmented. [15]
S. marcescens CMS515	CMS376 with *oxyR-2*, *oxyR*::pRMQS133 [15]
S. marcescens CMS519	CMS515 with restored wild-type *oxyR* [15]
S. marcescens CMS629	CMS376 with *fimC*::Tn-marniner [15]
S. marcescens Db10	Wild-type non-pigmented strain (19)
S. marcescens Db10 noChi	Db10 ΔchiA, ΔchiB, ΔchiC (19)

Fungal species (strain)	Phylum	Comments
Aspergillus fumigatus, a.f 293	*Ascomycota*	Our collection
Aspergillus flavus Afl1	*Ascomycota*	Our collection
Aspergillus nigerA20	*Ascomycota*	Our collection
Penicillium 304	*Ascomycota*	Beilinson hospital, Israel
Penicillium 4950	*Ascomycota*	Beilinson hospital, Israel
Penicillium 4956	*Ascomycota*	Beilinson hospital, Israel
Penicillium5475	*Ascomycota*	Beilinson hospital, Israel
Rhizopus microspores	*Zygomycota*	Beilinson hospital, Israel
Rhizopus oryzae 3465	*Zygomycota*	Beilinson hospital, Israel
Rhizopus oryzae 5571969	*Zygomycota*	Beilinson hospital, Israel
Rhizopus oryzae 5698	*Zygomycota*	Beilinson hospital, Israel
Rhizopus oryzae 579-19542	*Zygomycota*	Ichilov hospital, Israel
Rhizopus 579-1448	*Zygomycota*	Ichilov hospital, Israel
Absydia	*Zygomycota*	Ichilov hospital, Israel
Mucor circinelloides 103	*Zygomycota*	Ichilov hospital, Israel
Alternaria alternata	*Basidiomycota*	ATCC 1044
Cladosporium cladosporoides	*Basidiomycota*	ATCC 9836
Phanerochaete chrysosporium	*Basidiomycota*	HUJI Rehovot, Israel
Pleurotus ostreatus	*Basidiomycota*	ATCC 58053
Pleurotus pulmonarius	*Basidiomycota*	Inst. of Technical Microbiology, Jena, Germany
Schizophyllum commune	*Basidiomycota*	HUJI Rehovot, Israel

Ustilago sphaerogena	*Basidiomycota*	ATCC 12421
Gleophylum trabeum #512	*Basidiomycota*	University of Gottingen, Germany
Taramete versicolor #522	*Basidiomycota*	University of Gottingen, Germany
Lentinus lepideus #519	*Basidiomycota*	University of Gottingen, Germany
Ganoderma lucidum IMI109910	*Basidiomycota*	CABI Culture Collection

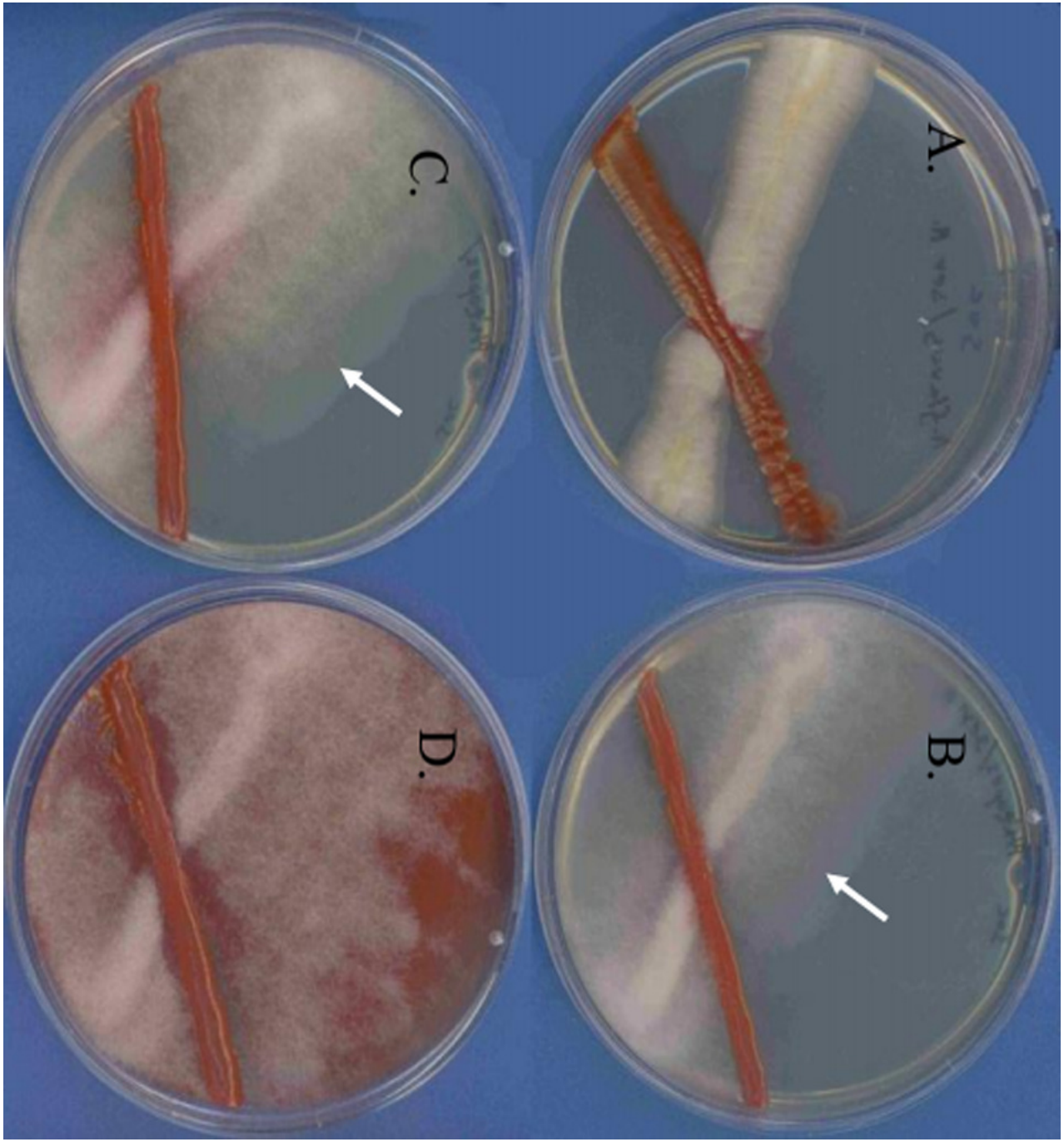
Table 2. Bacterial migration over aerial fungal hyphae*.

Bridging† after- Strain	48 h	96 h	Bacteria on aerial hyphae‡ 96 h
*R. oryzae 3465**	1/3	1/3	3/3
*R. oryzae 5698**	0/3	2/3	3/3

*The experiment was repeated three times for each bacterial-fungal combination to minimize the error rate.

†Bridging= bacteria were present on the agar block containing the fungus

‡Bacteria on aerial hyphae= the bacteria were present on the aerial hyphae covering the bridge between the agar block containing the bacteria and the agar block containing the mold.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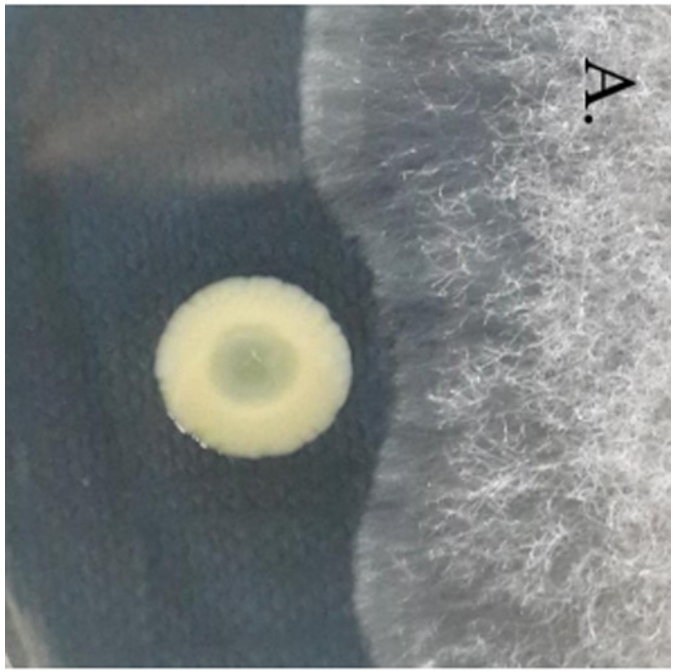
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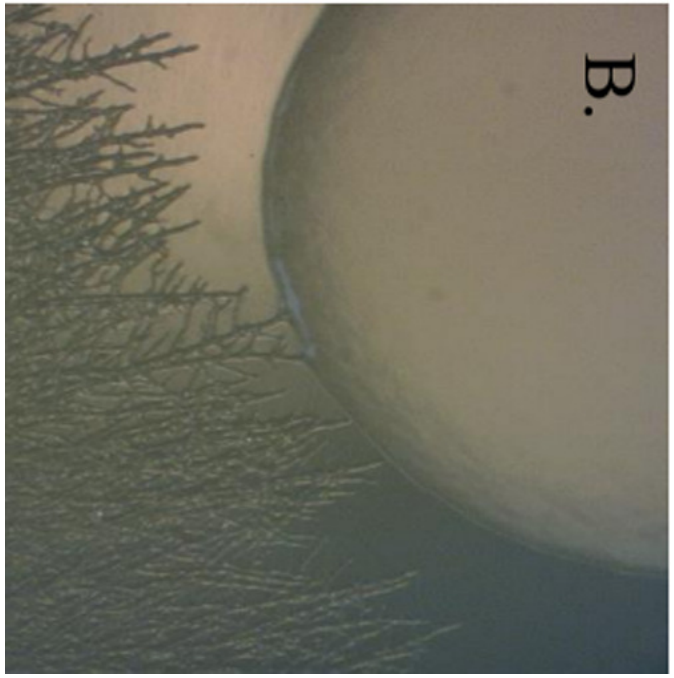
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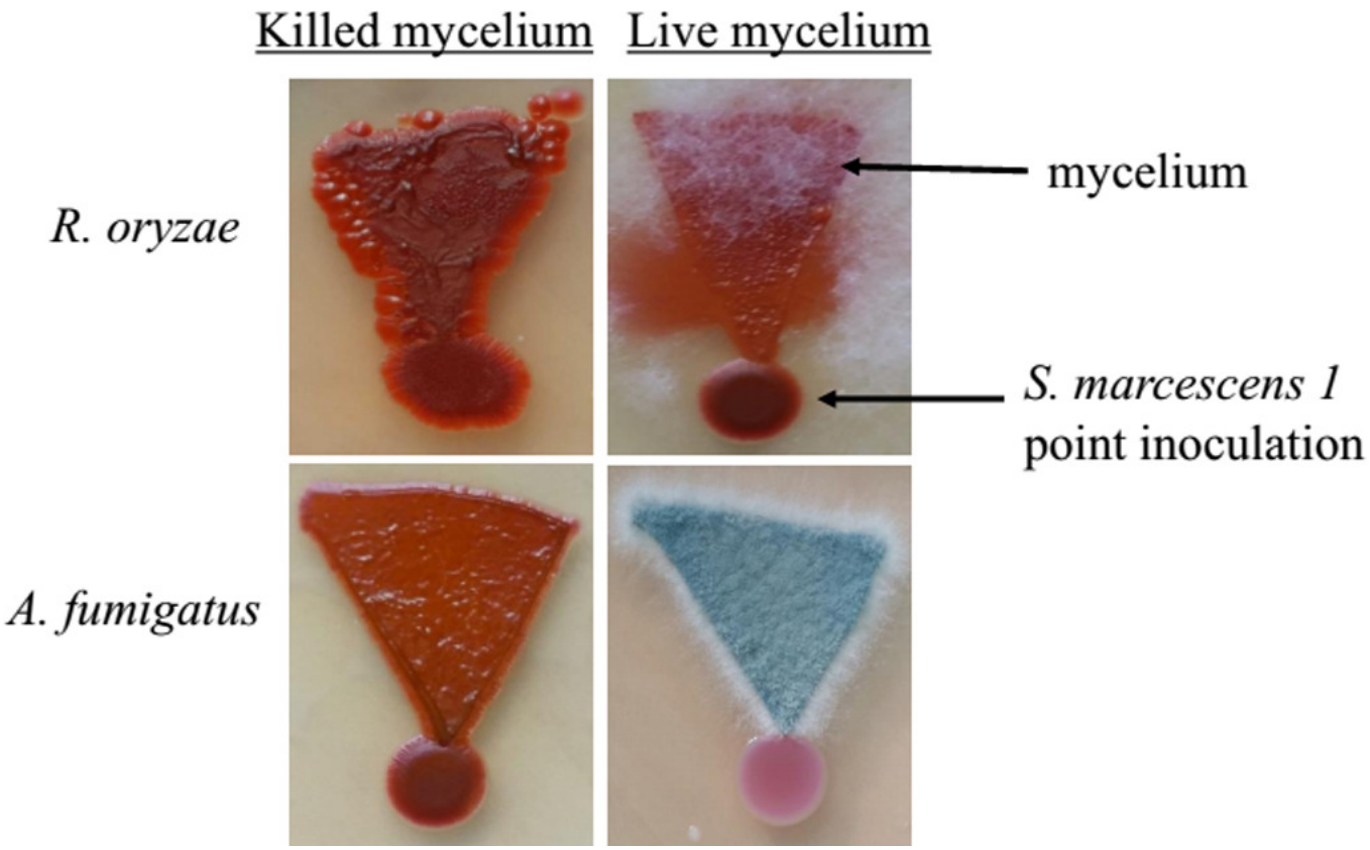

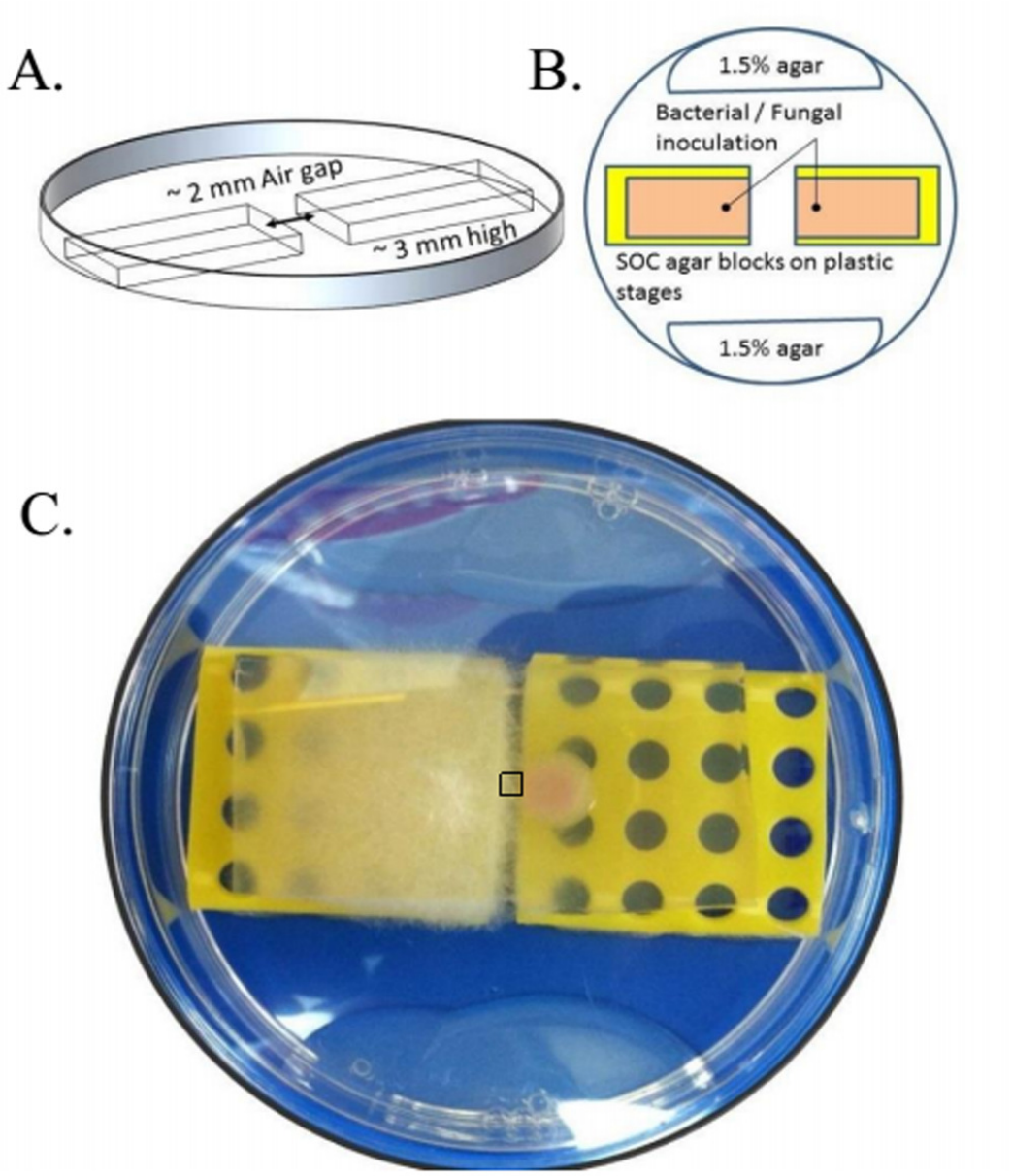

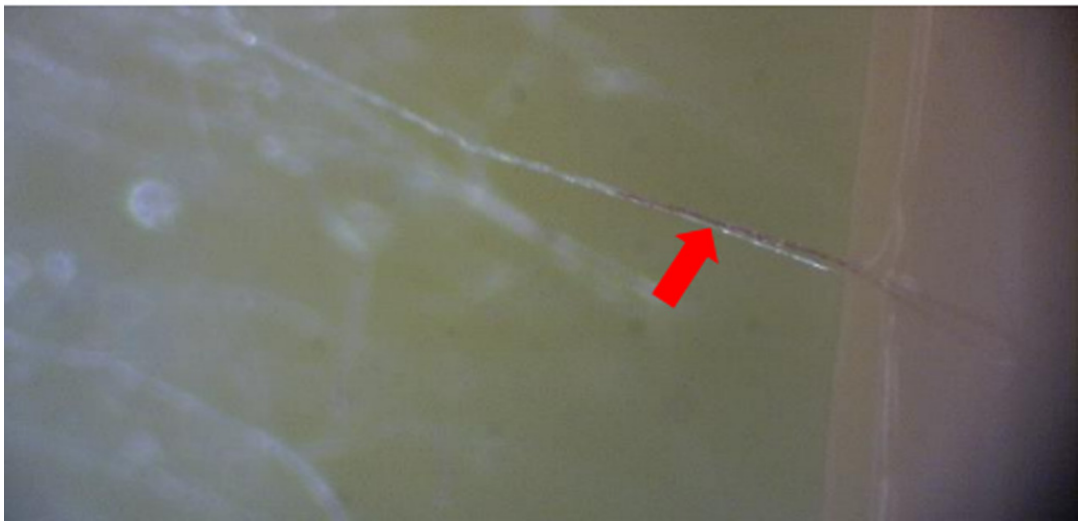

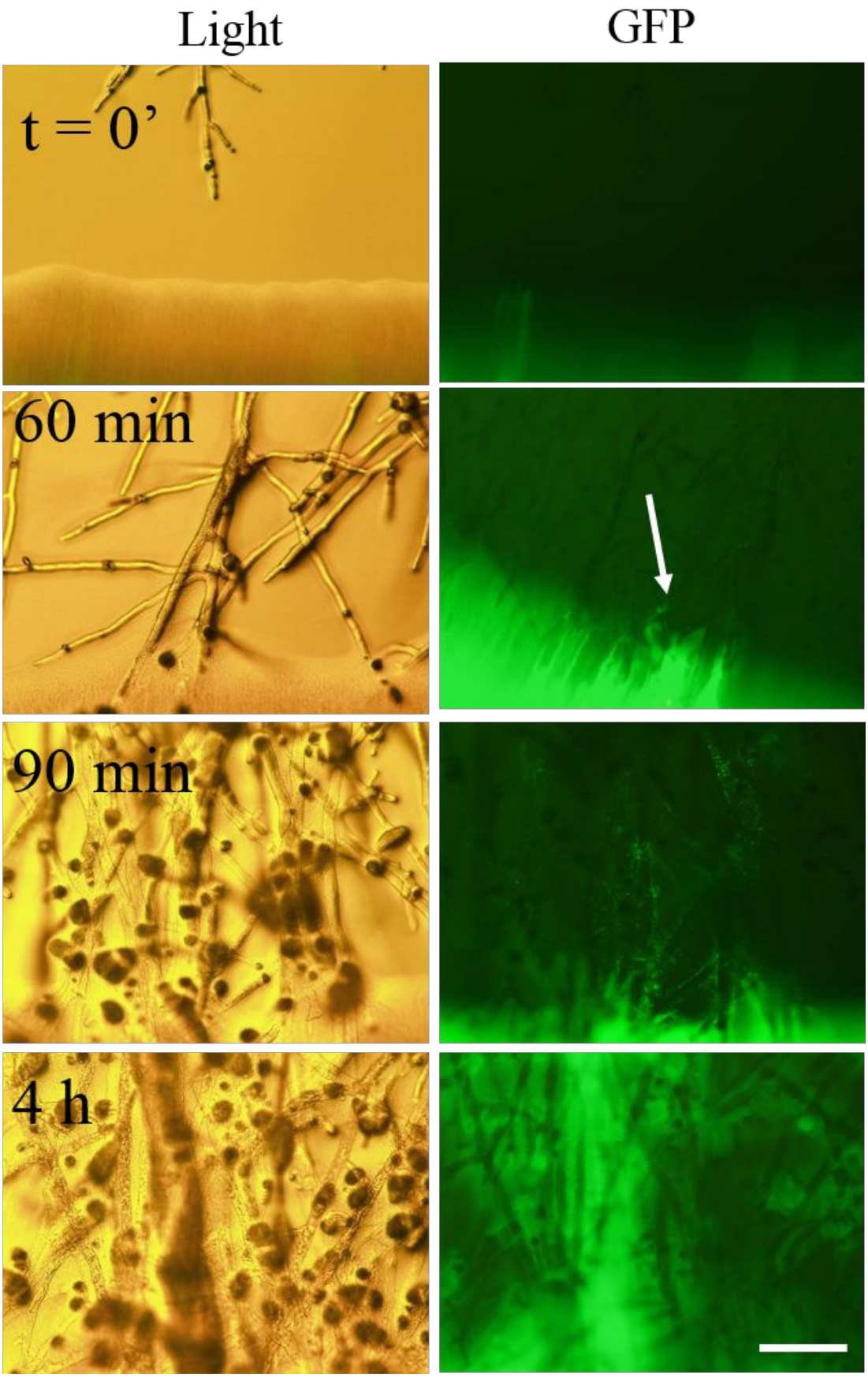

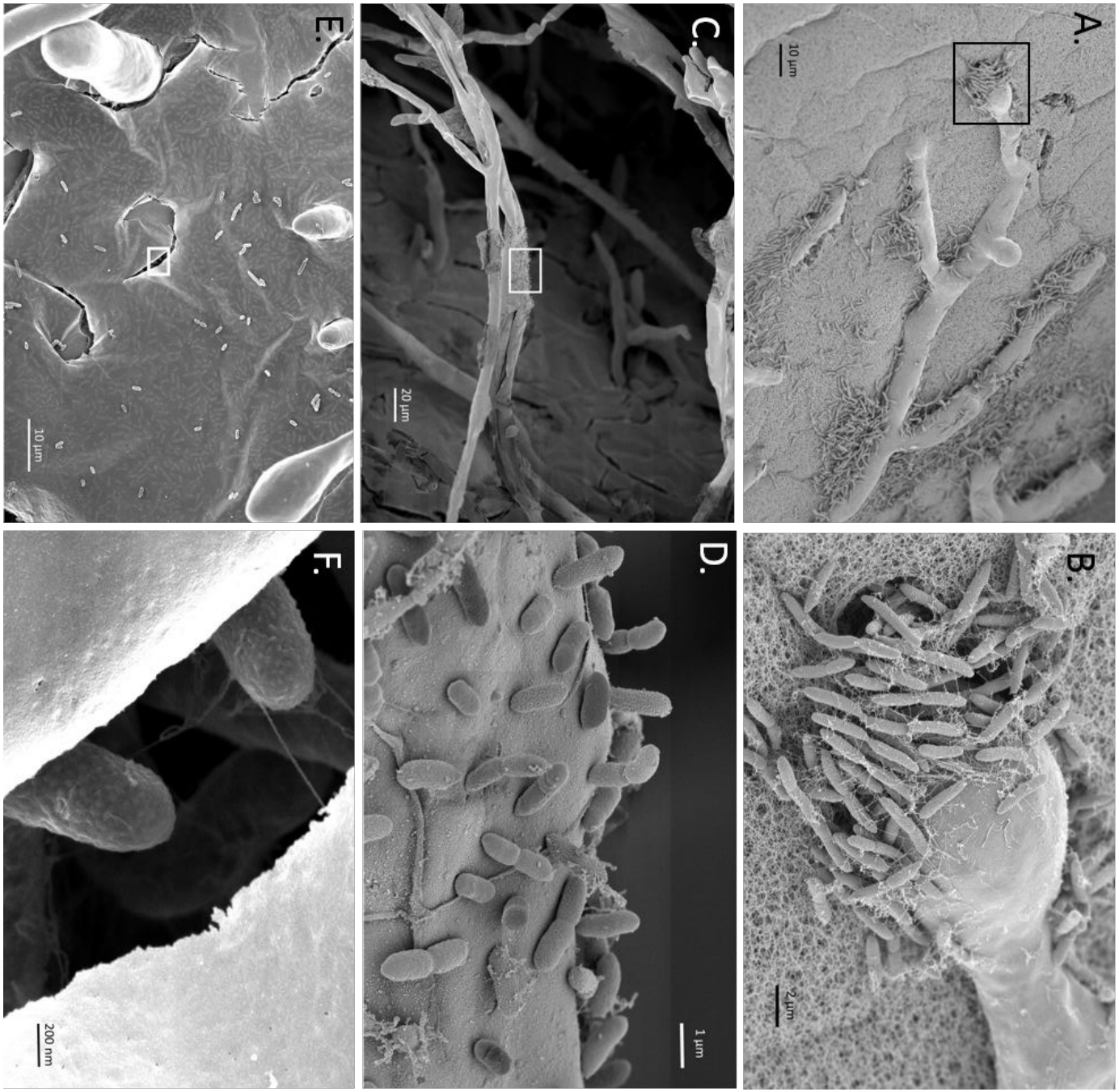
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