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Mechanisms of Bacterial (*Serratia marcescens*) Attachment to, Migration along, and Killing of Fungal Hyphae

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We have found a remarkable capacity for the ubiquitous Gram-negative rod bacterium *Serratia marcescens* to migrate along and kill the mycelia of zygomycete molds. This migration was restricted to zygomycete molds and several basidiomycete species. No migration was seen on any molds 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 toward 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 that covered and killed the mycelium. Flagellum-defective strains of *S. marcescens* were able to migrate along zygomycete hyphae, although they were 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 as 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. A better understanding of the mechanisms by which *S. marcescens* binds to, spreads on, and kills fungal hyphae might serve as an excellent model system for such interactions in general; fungal killing could be employed in agricultural fungal biocontrol.

Prokaryote-eukaryote interactions 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 and Kolter (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 hypha-attached bacterial colonies and then forms biofilm (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 nonsaturated 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 nonpigmented strain of *Serratia 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 composed primarily of chitin (12), which is susceptible to enzymatic breakdown by chitinases secreted by *S. marcescens* (10).

To define the complex interactions between the bacterium *S.*

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marcescens and fungi, we tested the hypotheses that the bacterium has the ability to use fungal hyphae as a means of translocation and that this ability is most significant when the bacterium is 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 a lack of spore formation, basidiomycete strains were cultured by seeding mycelial plugs ~2 cm^2 in size from a 2- to 5-day-old mycelium grown on YAG at 37°C. Mycelia were harvested with a sterile surgical blade under aseptic conditions.

The 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. The antibiotics used in the study were ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), gentamicin (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° angle on solid medium and incubated at 30 or 37°C for 48 h. Inoculation was performed by streaking a spore-bacterial suspension; the first streak was allowed to dry thoroughly prior to the next to avoid dragging the spores along the path of the bacteria. Bacterial spreading along the mycelium was assessed visually.

For nonsporulating basidiomycetes, mature mycelial plugs were seeded on an SOC plate, forming a line. After 2 to 5 days at 30°C, 10 μl of *S. marcescens* 1 or *S. marcescens* RM66262 suspension (OD, 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 an SOC plate at a concentration of 2.5×10^4 spores/cm^2, allowed to germinate for 12 h at 37°C, and then inoculated at the center of the plate by pipetting 5 μl of 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 the 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 postinoculation. 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 of SOC/well) at a concentration of 2.5×10^4 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 time periods indicated in Fig. 7B and 8B. 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 an 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.

TABLE 1 Bacterial and fungal strains used in this study

S. marcescens strain or fungal phylum, genus, species, and/or strain	Comment(s) (reference)
S. marcescens strains	
1	Environmental isolate, pigmented
RM66262	Wild-type clinical isolate, nonpigmented (25)
RM66262 GFP	RM66262/pBBR1-MCS2:promLac-GFP (20)
RM66262 Δ*flhD*	RM66262 *flhD*::pKNOCK-Cm (20)
RM66262 Δ*fliA*	RM66262 *fliA*::pKNOCK-Cm (16)
RM66262 Δ*shlB*	RM66262 shlB::pKNOCK-Sp (16)
RM66262 Δ*phlAB*	RM66262 *phlAB*::Cm (26)
CMS 376	Wild type, originally from Presque Isle culture (strain 3611), pigmented (15)
CMS 515	CMS 376 with *oxyR2*, *oxyR*::pRMQS133 (15)
CMS 519	CMS 515 with restored wild-type *oxyR* (15)
CMS 629	CMS 376 with *fimC*::Tn-*mariner* (15)
Db10	Wild-type nonpigmented strain (19)
Db10 no-Chi	Db10 Δ*chiA* Δ*chiB* Δ*chiC* (19)
Fungi	
Ascomycota	Our collection
Aspergillus fumigatus af293	Our collection
Aspergillus flavus Afl1	Our collection
Aspergillus niger A20	Our collection
Penicillium sp. strain 304	Beilinson Hospital, Israel
Penicillium sp. strain 4950	Beilinson Hospital, Israel
Penicillium sp. strain 4956	Beilinson Hospital, Israel
Penicillium sp. strain 5475	Beilinson Hospital, Israel
Zygomycota	
Rhizopus microspores	Beilinson Hospital, Israel
Rhizopus oryzae 3465	Beilinson Hospital, Israel
R. oryzae 5571969	Beilinson Hospital, Israel
R. oryzae 5698	Beilinson Hospital, Israel
R. oryzae 579-19542	Ichilov Hospital, Israel
Rhizopus sp. strain 579-1448	Ichilov Hospital, Israel
Absidia	Ichilov Hospital, Israel
Mucor circinelloides 103	Ichilov Hospital, Israel
Basidiomycota	
Alternaria alternata	ATCC 1044
Cladosporium cladosporioides	ATCC 9836
Phanerochaete chrysosporium	HUJI Rehovot, Israel
Pleurotus ostreatus	ATCC 58053
Pleurotus pulmonarius	Institute of Technical Microbiology, Jena, Germany
Schizophyllum commune	HUJI Rehovot, Israel
Ustilago sphaerogena	ATCC 12421
Gloeophyllum trabeum 512	University of Göttingen, Germany
Trametes versicolor 522	University of Göttingen, Germany
Lentinus lepideus 519	University of Göttingen, Germany
Ganoderma lucidum IMI109910	CABI Culture Collection

***S. marcescens* migration over dead fungal mycelium.** A total of 10^5 *R. oryzae* spores were grown in a petri dish containing 20 ml of 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 was frozen intact at −70°C and lyophilized. For preparation of the *Aspergillus fumigatus* mycelium, 10^5 *A. fumigatus* spores were grown in 100 ml of 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 for each side of the mycelium) on a Vilber Lourmat ECX-F15m UV table. Complete mycelial death was confirmed by placing the mycelia 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 and placing the pieces 2 to 3 mm apart from a bacterial colony made of 5 μl of logarithmic-phase (OD = 0.5) bacteria suspended in SOC. The 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 to 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 of a 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 *R. oryzae* spores and an *S. marcescens* 1 suspension at opposite sides, and incubated at 37°C for 48 to 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. A 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.

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 scanning electron microscopy (SEM) were performed, essentially as described previously (13). Briefly, 5- by 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 double-distilled water (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 using a K850 critical-point dryer (Quorum Technologies, United Kingdom). Coating was performed with 3 nm of iridium using a Q150T coater (Quorum Technologies). 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 mycelia of zygomycete molds isolated from the same microenvironment, whereas no spreading of *S. marcescens* over *Aspergillus flavus* mycelium or the surrounding agar surface was seen (Fig. 1). To better define this phenomenon, 26 fungal species representing three phyla (Table 1) were tested against one red-pigmented environmental strain of *S. marcescens* 1 and one nonpigmented patient-isolated strain (RM66262) at 30 or 37°C on 1.5% SOC agar. We decided to use two temperatures, since Soo 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 *Serratia* strains 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 *Gloeophyllum 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 cladosporioides*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Schizophyllum commune*, and *Trametes versicolor* (Table 1).

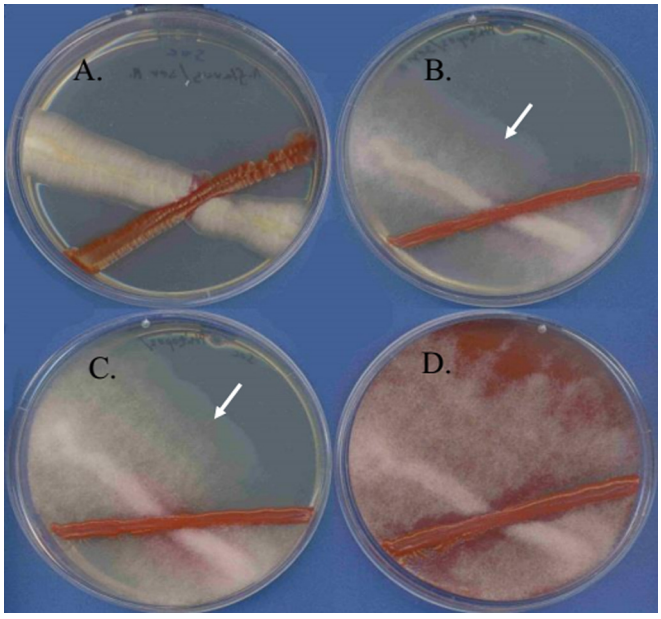

FIG 1 *S. marcescens* spreads over *Rhizopus oryzae* mold. *S. marcescens* 1 (red) was cocultured at a 45° angle to various mold species (white). (A) *Aspergillus flavus* AflI and *S. marcescens* 1 after 48 h of coincubation. No spreading of *S. marcescens* over *A. flavus* mycelium is seen. (B to D) *R. oryzae* and *S. marcescens* 1 after 24 h (B), 30 h (C), and 48 h (D) of incubation. 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.

***S. marcescens* does not exhibit active chemotaxis 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 toward 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 of 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 to 3 mm from the edge of UV-killed and lyophilized *R. oryzae* 3465 or *A. fumigatus* mycelium. As controls, we used live mycelia. As described above, live *R. oryzae* my-

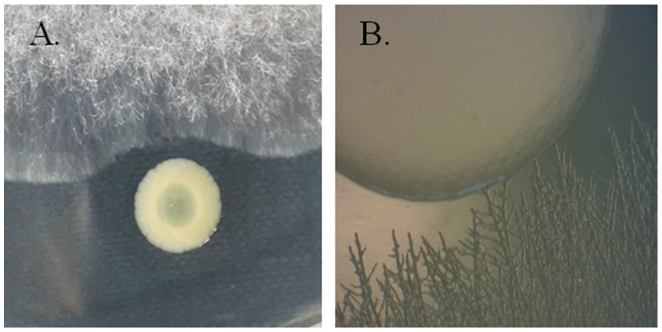

FIG 2 *S. marcescens* strain RM66262 does not show chemotaxis toward *R. oryzae* 5698. Bacterial and fungal strains were point inoculated adjadently. (A) No visible change in bacterial colony morphology is observed, indicating that there is no chemotaxis. (B) *S. marcescens* migration begins only after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

celium 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. Both dead *R. oryzae* and *A. fumigatus* mycelia were entirely covered by bacteria after 36 h of 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, and (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 directly across aerial hyphae 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 and the other with *R. oryzae* 3465 or *R. oryzae* 5698, separated by an aerial gap (Fig. 4A to C). Movement between the isolated blocks is possible only via the bridge of aerial hyphae formed between them after ~48 h of incubation at 37°C in a humid chamber. Successful bacterial "bridging" was defined by

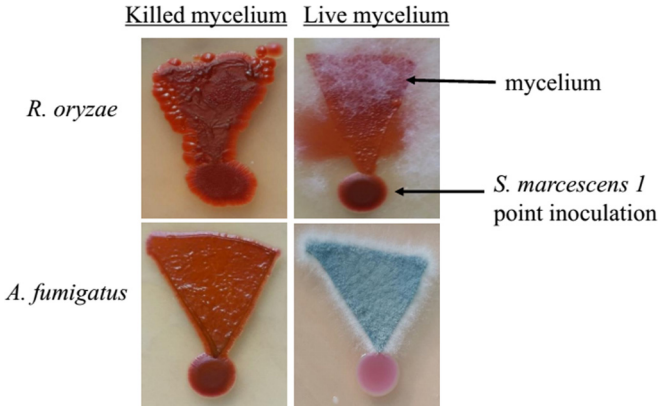

FIG 3 *S. marcescens* 1 migrates over killed mycelium of *R. oryzae* 3465 and *A. fumigatus* but fails to migrate on live *A. fumigatus* mycelium. Following 36 h of incubation at 30°C on SOC plates, red bacterial coloration is visible on the entire mycelial surface and edges of live or killed *R. oryzae*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

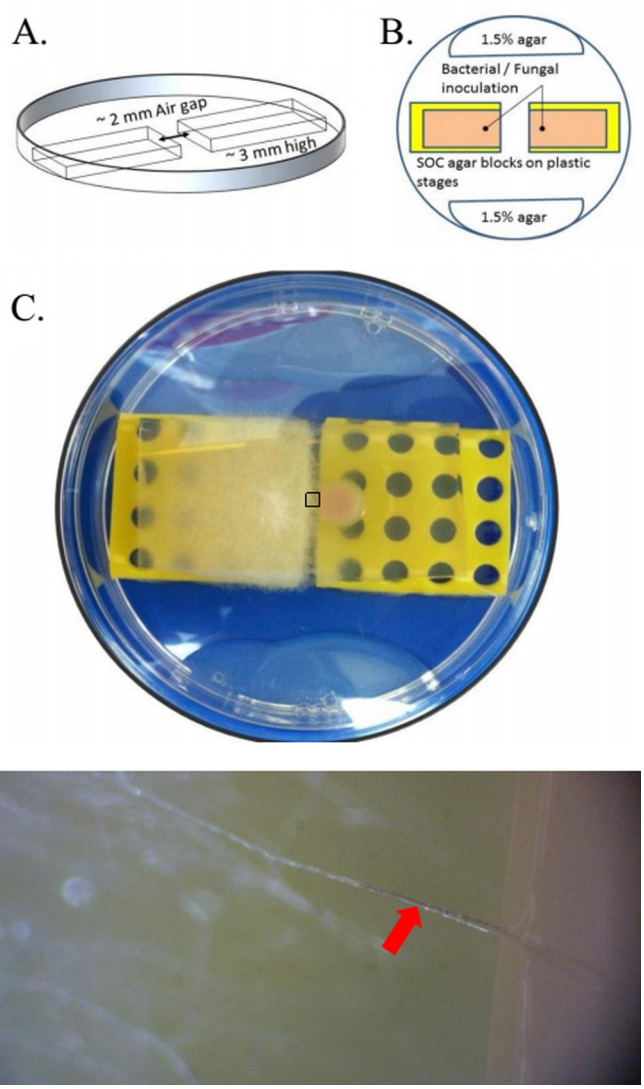

FIG 4 *S. marcescens* 1 crosses an aerial bridge of *R. oryzae* 3465 hyphae. (A and B) Scheme of bridging experiment plate, containing two separate raised SOC agar blocks (orange in panel B) inoculated with bacterial and fungal inoculum separately and incubated in a humid chamber. To eliminate bacterial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

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. 4C, bottom).

Microscopic analysis of *S. marcescens* movement along *R. oryzae* hyphae on solid medium by fluorescence and SEM. For fluo-

TABLE 2 Bacterial migration over aerial fungal hyphae[a]

R. oryzae strain	No. of expts (out of 3) with: Samples that bridged[b] after: 48 h	96 h	Bacteria on aerial hyphae at 96 h[c]
3465	1	1	3
5698	0	2	3

[a] The experiment was repeated three times for each bacterial-fungal combination to minimize the error rate.
[b] Bridging indicates that the bacteria were present on the agar block containing the fungus.
[c] Bacteria present on the aerial hyphae covering the bridge between the agar block containing the bacteria and the agar block containing the mold.

rescence 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 fluorescence 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 min). 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 (Fig. 6A to D) and the subsequent formation of a mature biofilm covering the mycelium (Fig. 6E and 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, the motility of *S. marcescens* RM66262 strains with deletions of *flhD* (lacking $FlhD_2C_2$, the flagellar master regulator operon) and *fliA* (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 that of the *S. marcescens* RM66262 isogenic wild type. Spreading motility was measured on a mycelium-covered plate (*R. oryzae* 3465), as described in Materials and Methods. Our results show that spreading of the Δ*fliA* and Δ*flhD* mutants was slower than that of the wild type (Fig. 7A). Interestingly, the killing abilities of the *S. marcescens* Δ*fliA* and Δ*flhD* mutants were diminished (Fig. 7B). Whereas the wild-type *S. marcescens* strain killed *R. oryzae* 3465 after 36 h of coinoculation, 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 Materials 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 might be responsible for the reduced killing observed for the Δ*flhDC* and Δ*fliA* mutant strains. Moreover, because the levels of expression of the pore-forming toxin ShlA (encoded by the *shlBA* operon) were previously shown to be controlled by the FlhDC-FliA regulatory cascade (16, 17), we also assayed the Δ*shlB* mutant strain for its *R. oryzae* 3465 killing capacity. However, there were no differences in the killing values of the Δ*phlA* or Δ*shlB* mutant strains from that of the wild-type strain (data not shown), indicating that the altered expression of these secreted enzymes in the Δ*flhD* or Δ*fliA* mutant strains is not responsible for the observed attenuated killing phenotype, although we cannot rule out the possibility that the deletion of Δ*phlA* and Δ*shlB* together may contribute synergistically to fungal killing.

***S. marcescens* strains defective in fimbrial expression migrate faster along *R. oryzae* 3465 mycelia than strains that are not defective.** Type 1 fimbriae 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

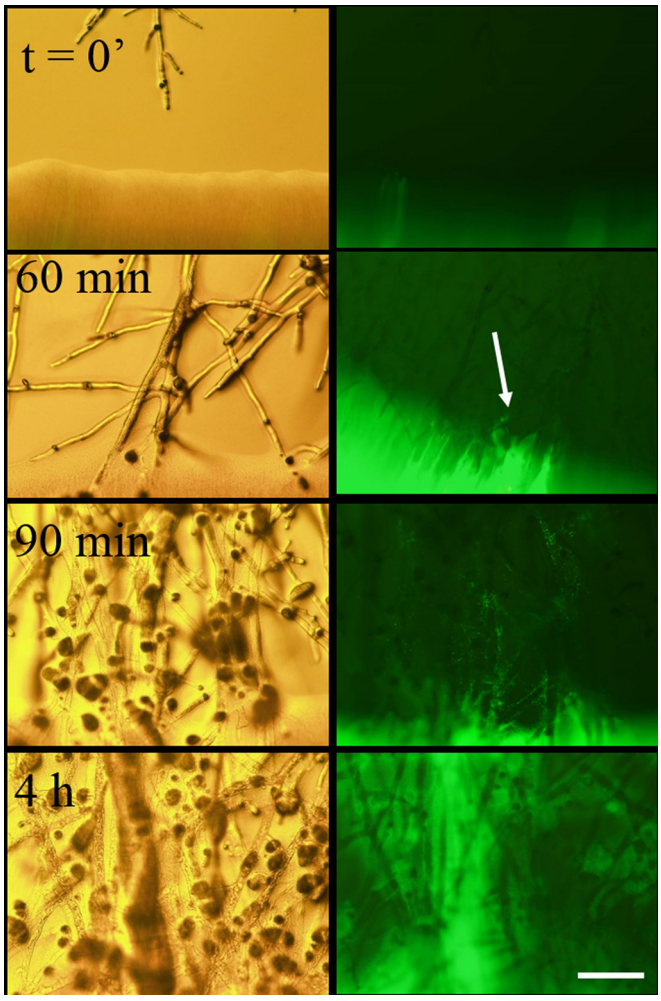

FIG 5 Fluorescence microscopy of *S. marcescens* interacting with *R. oryzae* mycelium. *S. marcescens* 66262/pGFP and *R. oryzae* 3465 were inoculated adjacently on SOC agar plates and incubated at 37°C until the fungal hyphae came into physical contact with the bacterial colony (time zero [t = 0′]). Light and fluorescent (green fluorescent protein [GFP]) microscopy were performed on the contact region at different time points. The arrow indicates initial early colonization of hyphae by *S. marcescens* bacteria after 60 min of interaction. Scale bar = 50 μm.

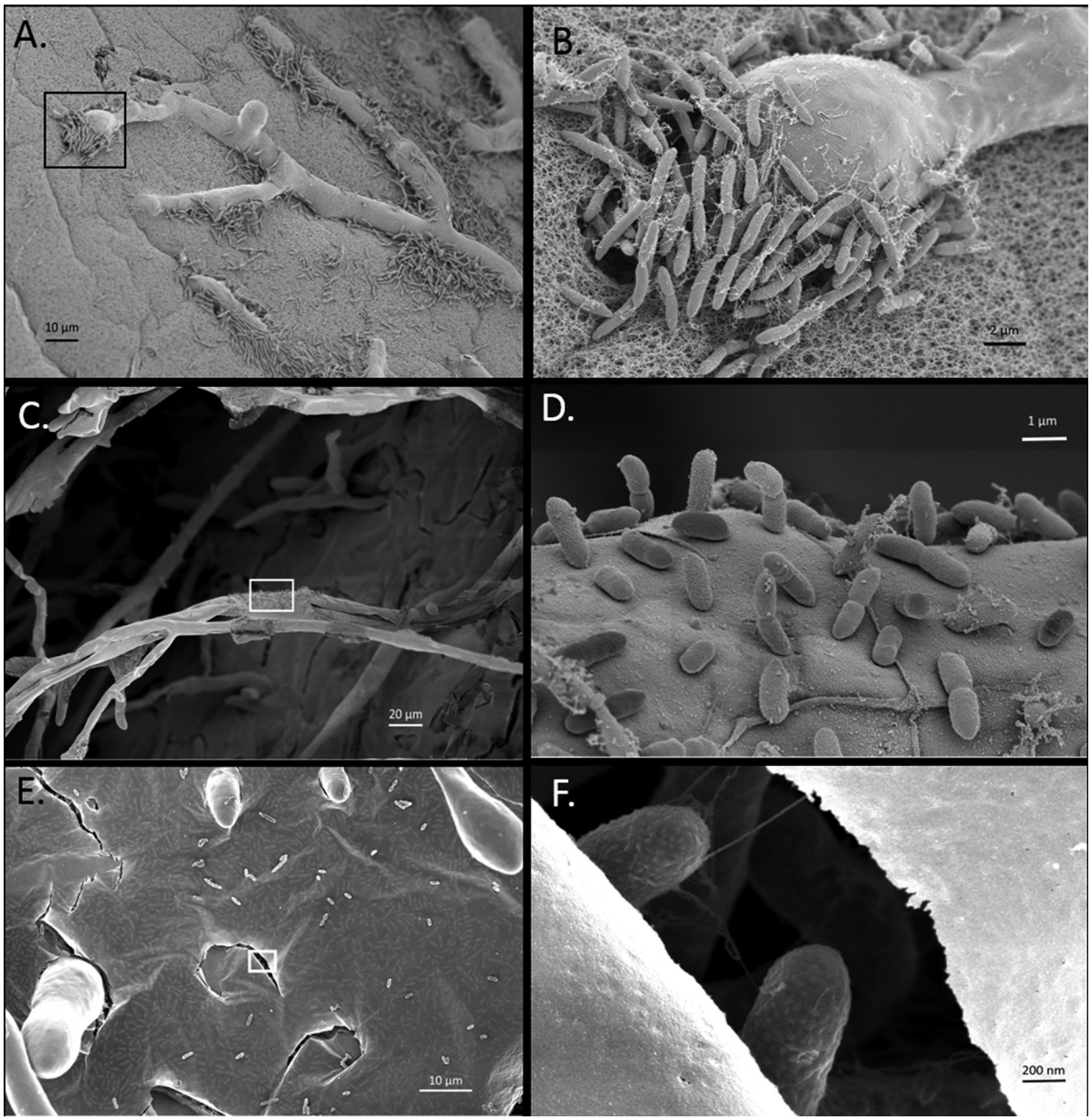

FIG 6 High-resolution SEM imaging of *S. marcescens* interacting with *R. oryzae* mycelium. *S. marcescens* RM66262 forms discrete colonies on *R. oryzae* 3465 hyphae during early (1 to 2 h) contact on the surface of SOC agar plates (A; the inset in panel A is enlarged in panel B), on aerial hyphae (C;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

(contains a *fimC* mutant usher gene required for fimbria formation) to those of the CMS 376 wild type and CMS 519 (the *oxyR2* mutant revertant that generates fimbriae 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 fimbriae 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 activities of a no-Chi (Δ*chiA* Δ*chiB* Δ*chiC*) triple mutant lacking any detectable secreted chitinase activity to those 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 from that of 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.

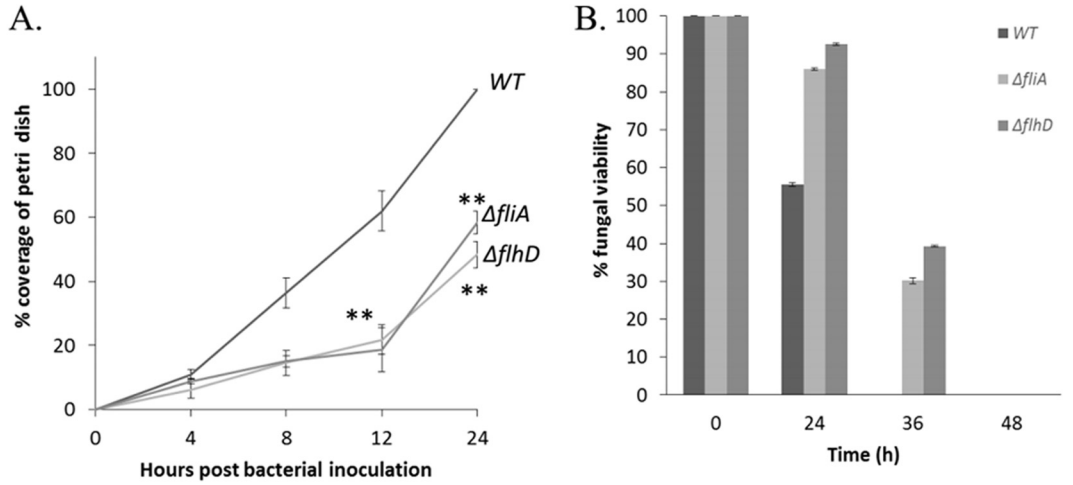

FIG 7 *S. marcescens* mutants lacking a flagellum show impaired migration and killing of *R. oryzae* 3465. (A) To measure bacterial spreading, the *S. marcescens* RM66262 wild type (WT), Δ*flhD* mutant (defective in the flagellar master regulator), and Δ*fliA* mutant (defective in the flagellar class 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 1.5-cm SOC 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 the plates for fungal growth, as described in Materials and Methods. The data are representative of the results from three experiments; shown are averages ± standard deviations (SD) from triplicate samples. **, $P < 0.005$ between mutant and WT strains.

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 the 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 mycelia 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 10 species enabling strong migration, while others allowed only peripheral migration or none at all. These results can be explained by two alternative nonexclusive 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, and bacterial attachment and movement occur only on fungi possessing these determinants; (ii) some fungal species/phyla produce surface or secreted defense factors that block bacterial interactions, and bacterial attachment and movement occur 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

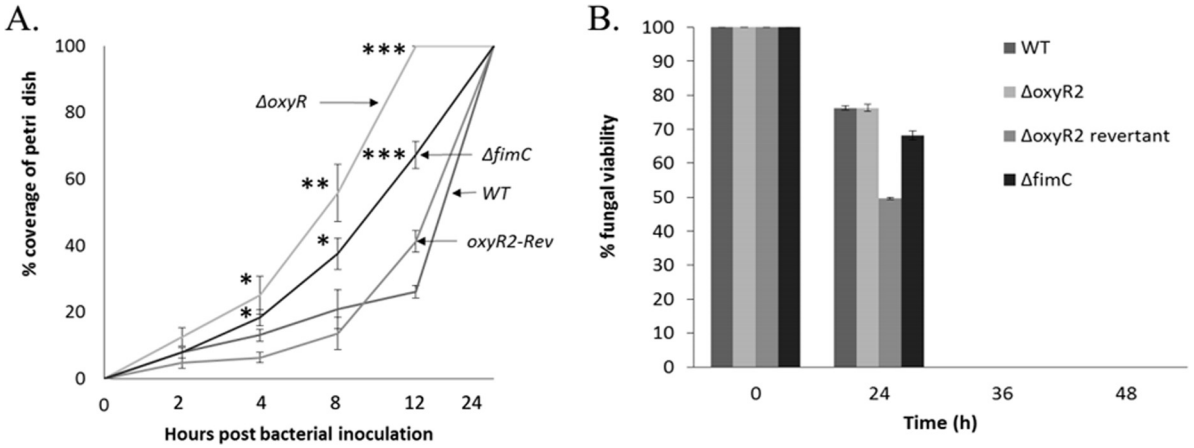

FIG 8 Fimbria-defective *S. marcescens* mutants migrate faster than the wild type along *R. oryzae* hyphae. (A) Migration along a preformed *R. oryzae* 3465 mycelium by the *S. marcescens* CMS 376 wild type, Δ*oxyR2* mutant (defective in the oxidative-stress reaction and fimbrial expression), and Δ*fimC* mutant (defective in fimbrial structural genes). Strains were inoculated separately on the center of an *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 CMS 376 wild-type strain. The data are representative of the results from three experiments; shown are averages ± SD from triplicate samples. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ between mutant and WT strains.

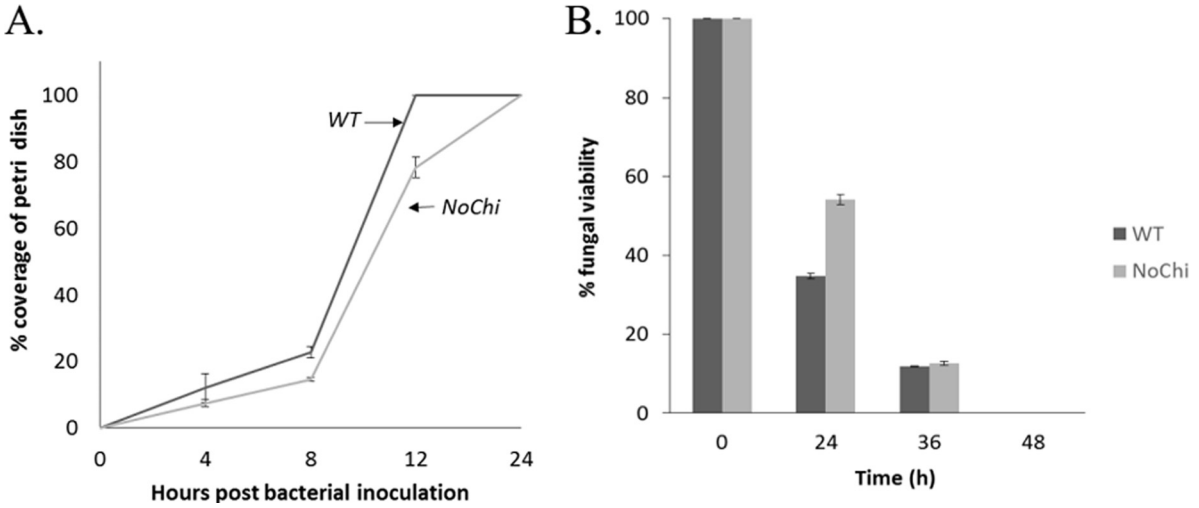

FIG 9 *S. marcescens* migration and killing of *R. oryzae* 3465 do not depend on its ability to secrete chitinases. (A) To measure bacterial spreading, the *S. marcescens* Db10 wild type and Db10-NoChi strain (lacking detectable secreted chitinase activity) were inoculated separately in the center of 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 the bacterial migration rate. (B) To measure hyphal killing, *S. marcescens* strains were inoculated in the center of 1.5-cm SOC 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 the plates for fungal growth, as described in Materials and Methods. The data are representative of the results from three experiments showing the averages ± SD from triplicate samples.

mycelium secretes factors that block bacterial interaction and migration, supporting the second hypothesis, at least for *A. fumigatus*. The identity of these bacterium-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 bacterium-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* (carrying 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 coincubation. These results might be explained by the existence of an additional non-flagellum-dependent mode of transportation that enables slower movement along hyphae, such as sliding, which is 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 might be due to the fact that the flagellum also functions as a secretion system with structural and functional relatedness to the type III secretion system. The flagellum is able to translocate the components involved not only in self-assembly but also in the secretion of nonflagellar proteins (23). Nonflagellar 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 the possibility that another yet-unknown protein(s) that might be transcriptionally dependent on the flagellar regulatory cascade or secreted by the flagellar secretome exerts 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* mutants) spread faster during the first 8 to 12 h. 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 interbacterial 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 hypothe-

sized 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 an *S. marcescens* triple-deletion mutant (Δ*chiA* Δ*chiB* Δ*chiC*) 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*-zygomycete interactions that 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 flagellum-defective *S. marcescens* strains were delayed in spreading and killing of the fungus, similar *P. aeruginosa* mutants showed normal killing. While pilus-deficient *S. marcescens* strains 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*-zygomycete 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, multigenetic, and multifactorial system. The complexity of the interaction, which takes place on solid agar surfaces and not in suspension in liquid medium, makes 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.

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