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Metschnikowia Strains Isolated from Botrytized Grapes Antagonize Fungal and Bacterial Growth by Iron Depletion▽

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Noble-rotted grapes are colonized by complex microbial populations. I isolated pigment-producing *Metschnikowia* strains from noble-rotted grapes that had antagonistic activity against filamentous fungi, yeasts, and bacteria. A red-maroon pigment was formed from a diffusible colorless precursor released by the cells into the medium. The conversion of the precursor required iron and could occur both in the cells (red colonies) and in the medium (red halos around colonies). The intensity of pigmentation was correlated with the intensity of the antimicrobial activity. Mutants that did not form pigment also lacked antifungal activity. Within the pigmented halos, conidia of the sensitive fungi did not germinate, and their hyphae did not grow and frequently lysed at the tips. Supplementation of the medium with iron reduced the size of the halos and the inhibition zones, while it increased the pigment accumulation by the colonies. The iron-binding agent tropolone had a similar effect, so I hypothesize that pigmented *Metschnikowia* isolates inhibit the growth of the sensitive microorganisms by pigment formation, which depletes the free iron in the medium. As the pigment is a large nondiffusible complex produced in the presence of both low and high concentrations of ferric ions, the proposed mechanism is different from the mechanisms operating in microbes that release siderophores into the environment for iron acquisition.

The important problems for postharvest protection of fruit include the declining effectiveness of registered fungicides, public pressure to reduce fungicide use, and public demand for produce free of synthetic pesticides. One solution is to use microorganisms with antifungal effects as biocontrol agents to reduce or inhibit the rate of propagation of destructive fungi during storage. Antagonistic yeasts have received particular attention, as their activity usually does not depend on the production of antibiotics or other toxic secondary metabolites. For example, strains of *Candida*, *Cryptococcus*, *Debaromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Sporobolomyces*, and *Trichosporon* all have been reported to inhibit postharvest decay of fruit due to their antifungal effects (6, 18, 36, 38, 40, 44, 50, 52). The modes of action proposed for the inhibition process include competition for space and nutrients, parasitism, direct interaction with the pathogen, production of cell wall lytic enzymes, and induced resistance in the host tissue (for a review, see reference 47).

Fruit-borne strains of *Metschnikowia pulcherrima* can be effective in protecting apples, peaches, and grapes against postharvest rot caused by *Botrytis cinerea* and other postharvest pathogens (14, 36, 48). The related species *Metschnikowia fructicola* is an effective biocontrol agent for postharvest diseases of grapes (25). *M. pulcherrima*, but not *M. fructicola*, produces a red pigment, pulcherrimin, that accumulates in the cells and in the medium near a colony (26, 27). Pulcherrimin is a large complex formed nonenzymatically from a dibasic acid, pulcherriminic acid, and ferric ions (11, 29). The mechanism of the antifungal antagonism and its relationship to the production of pigment have not been studied yet.

M. pulcherrima is common on wine grapes at the time of harvest (for a review, see reference 20) and in grape must during the early stages of wine fermentation (9, 10, 19, 31). *M. pulcherrima* occurs more frequently on damaged berries (37), on berries used to produce ice wine (7), and in botrytized (noble-rotted) wines (1). Ice wine is a late-harvest wine produced from grapes left on the vine until the first frost hits. These grapes are overripe and frequently rupture and partially desiccate before harvest. Noble rot of grapes occurs when the berries are infected by *B. cinerea*. Water evaporates through the *Botrytis*-generated skin lesions, and the grapes desiccate, resulting in high levels of sugar. The ability of *M. pulcherrima* to survive under these low-water-availability conditions (41) may be a reason for its prevalence on noble-rotted and ice wine grapes.

The objectives of this study were (i) to isolate pigment-producing strains of *Metschnikowia* from noble-rotted grapes, (ii) to test these isolates to determine their ability to antagonize the growth of filamentous fungi, yeasts, and bacteria, (iii) to determine the role of pigment production in the antagonism observed, and (iv) to identify the cytological target(s) of the inhibitory agent. Since pulcherrimin is a nondiffusible complex (11, 26, 29), I propose that *Metschnikowia* inhibits the growth of other microbes by immobilizing iron in the medium. This possibility has not been considered in previous reports on the antifungal antagonism of *Metschnikowia* strains. The proposed mechanism of iron depletion is different from the mechanisms operating in microbes that release siderophores (low-molecular-weight, ferric ion-specific chelators) into the environment for the purpose of absorbing iron (for a review, see reference 24). As iron is essential for the growth of many microorganisms

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TABLE 1. List of strains[a]

Strain	Source or reference
Aspergillus clavatus IMI 15949	UKNCC
Aspergillus niger IMI 17454	UKNCC
Aureobasidium pullulans 1.3.11	This study
Botrytis cinerea 980	22
Botrytis cinerea 3318	This study
10-432 *Candida stellata* CBS 157^T	CBS
10-372 *Candida zemplinina* CBS 9494^T	45
Escherichia coli DH5	Invitrogen
Gilbertella persicaria IMI 101638	UKNCC
10-511 *Hanseniaspora uvarum* CBS 314	CBS
Metschnikowia pulcherrima isolates 02.4.3.38 (= NCAIM Y0177071), 02.11.1.21 (= NCAIM Y0177072), 11.2.38 (= NCAIM Y0177071), 4a.3.11, 7.3.10, 7.3.37, 9.4.60, 17.1.IV, 17.3.1	This study
Metschnikowia pulcherrima mutants A-1 (isolated from 02.11.1.21), A-2 (isolated from 02.11.1.21), B-1 (isolated from 02.11.1.21), B-2 (isolated from 02.11.1.21), C (isolated from 02.11.1.21), D (isolated from 02.11.1.21), E (isolated from 02.11.1.21), F (isolated from 02.11.1.21), G-21 (isolated from 02.11.1.21), G-38 (isolated from 02.4.3.38), H-1 (isolated from 02.4.3.38), H-2 (isolated from 02.4.3.38)	This study
Mucor piriformis NRRL 3636	NRRL
Mucor circinelloides ATCC 1216	ATCC
Oenococcus oeni B16	Faculté d'Oenologie
Rhizopus stolonifer var. *stolonifer* CBS 109.76	CBS
10-157 *Saccharomyces cerevisiae* s288c	YGS
10-408 *Saccharomyces uvarum*	2
7-1 *Schizosaccharomyces japonicus* var. *japonicus* CCY-44-5-1	CCY
0-1 *Schizosaccharomyces pombe* var. *pombe* L972	Bern Collection
0-39 *Schizosaccharomyces pombe* var. *pombe leu1*	Bern Collection

[a] Abbreviations: ATCC, American Type Culture Collection, Manassas, Va.; Bern Collection, Institut für Allgemeine Mikrobiologie, Universität Bern, Bern, Switzerland; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; CCY, Czechoslovak Collection of Yeasts, Institute of Chemistry, Bratislava, Slovakia; Faculté d'Oenologie, Faculté d'Oenologie de Bordeaux UMR1219, Talence, France; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary; NRRL, National Center for Agricultural Utilization Research, Peoria, Ill.; UKNCC, United Kingdom National Culture Collection; YGS, Yeast Genetic Stock, Berkeley, Calif. Numbers 0-1, 0-39, 7-1, 10-157, 10-372, 10-408, 10-432, and 10-511 are codes used for the strain collection of the Department of Genetics, University of Debrecen (Hungary).

and microbial pathogenesis, iron sequestration by nonpathogenic microbes can be exploited in novel and more environmentally benign systems for postharvest protection against destructive fungi.

MATERIALS AND METHODS

Organisms, media, and chemicals. All fungal strains (Table 1) were maintained on potato dextrose agar (PDA) (49). For production of conidia, strains were grown on PDA at 15 to 20°C for 1 week with daylight (on a laboratory bench in an air-conditioned room). Conidia were harvested by washing the mycelium with 10 ml of sterile distilled water. Yeast strains were grown on yeast extract peptone agar (YPD) (49) or in liquid YPDL medium (YPD without agar) at 30°C. *Oenococcus oeni* was cultured on MRS medium (16) at 30°C. *Escherichia coli* was maintained on LB agar (42) at 37°C. Synthetic minimal agar (SMA), SML (SMA without agar), yeast extract agar (YEA), and YEL (YEA without agar) (46) were used in pigment production tests. The sensitivity of strains to iron depletion was tested with tropolone (2-hydroxycyclohepta-2,4,6-trienone; T7387; Sigma-Aldrich Co., St. Louis, Mo.), a chelating agent with a strong affinity for ferric ions (12, 17).

Strain isolation and taxonomic identification. Grape berries were collected aseptically from noble-rotted bunches. Each berry was homogenized separately in 2 ml of sterile water, and samples of the resulting must were spread on YPD plates. After incubation at room temperature (15 to 20°C) for 5 days, red or reddish colonies fringed by reddish halos in the medium were isolated. Each isolate was spread once or twice on YPD plates to obtain clones originating from single cells. Morphological examination and tests for carbon and nitrogen source utilization for species identification were conducted as previously described (4, 49). Ten colonies of *Aureobasidium pullulans* and 10 colonies with *Botrytis* morphology also were recovered. The sequences of the D1/D2 domains at the 5′ end of the large-subunit 26S rRNA genes of the yeast isolates and the *Botrytis* isolates were amplified with primers NL-1 and NL-4 (35) by using PCR conditions described previously (45). Both strands of the fragments were sequenced with an ABI PRISM 3700 sequencer (AME Bioscience Ltd., Sharnbrook, United Kingdom) by using the PCR primers. Sequence similarity searches were performed with the BLAST network service of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Tests for pigment production. To compare pigment production in colonies growing on agar plates, cells of the isolates either were streaked on agar plates or were suspended in sterile water ($\sim 10^7$ cells per ml), and 15-μl samples of the suspensions were dropped onto the surfaces of agar plates. The widths of the reddish halos around the yeast colonies were measured after 5 to 10 days of incubation at 25°C. Pigment production also was tested in shake cultures at 25°C (50 ml medium in a 200-ml Erlenmeyer flask; incubation at 100 rpm in a gyratory shaking incubator for 14 h; inoculum, 10^4 cells/ml).

Tests for antagonism. Inhibition of conidial germination was examined on PDA plates flooded with suspensions of conidia. One yeast isolate was streaked or dropped on each plate and incubated at 25°C. To examine the inhibition of hyphal growth, spores were streaked on PDA plates and incubated for 2 days at 25°C to allow them to develop mycelia. The yeast isolates tested were inoculated as colonies from 15-μl spots placed 5 mm ahead of the growing edges of the mycelia. The plates were checked for inhibition twice a day for 7 days because the growth rates of the fungal strains differed.

The effects of *Metschnikowia* isolates 02.11.1.21 and 02.4.3.38 on yeasts and bacteria were tested on plates flooded with cells of the test organisms. The media used were PDA for yeasts, MRS agar for *O. oeni*, and LB agar for *E. coli*. One yeast isolate was streaked or dropped on each plate. The incubation temperatures were 25°C for yeasts, 30°C for *O. oeni*, and 37°C for *E. coli*.

Effect of iron and tropolone on pigment production and antagonism. The effect of ferric ions on pigment production was studied on PDA plates supplemented with $FeCl_3$. The plates were flooded with conidia. One yeast isolate was then inoculated into the center of each plate. The sensitivity of conidial germination to tropolone was tested by placing 50-μl samples of aqueous solutions of the compound into wells (diameter, 5 mm) cut into PDA plates previously flooded with suspensions of *Botrytis* conidia. The widths of the pigmented halos and inhibition zones were measured after 3 to 10 days of incubation at 25°C.

Mutagenesis and mutant characterization. Yeast cells in overnight YPDL cultures (50 ml medium in a 200-ml Erlenmeyer flask; incubation at 100 rpm in a gyratory shaking incubator for 14 h at 25°C; inoculum, 10^4 cells/ml) were collected by centrifugation (1,500 × *g*, 5 min, 10°C), resuspended in fresh YPDL

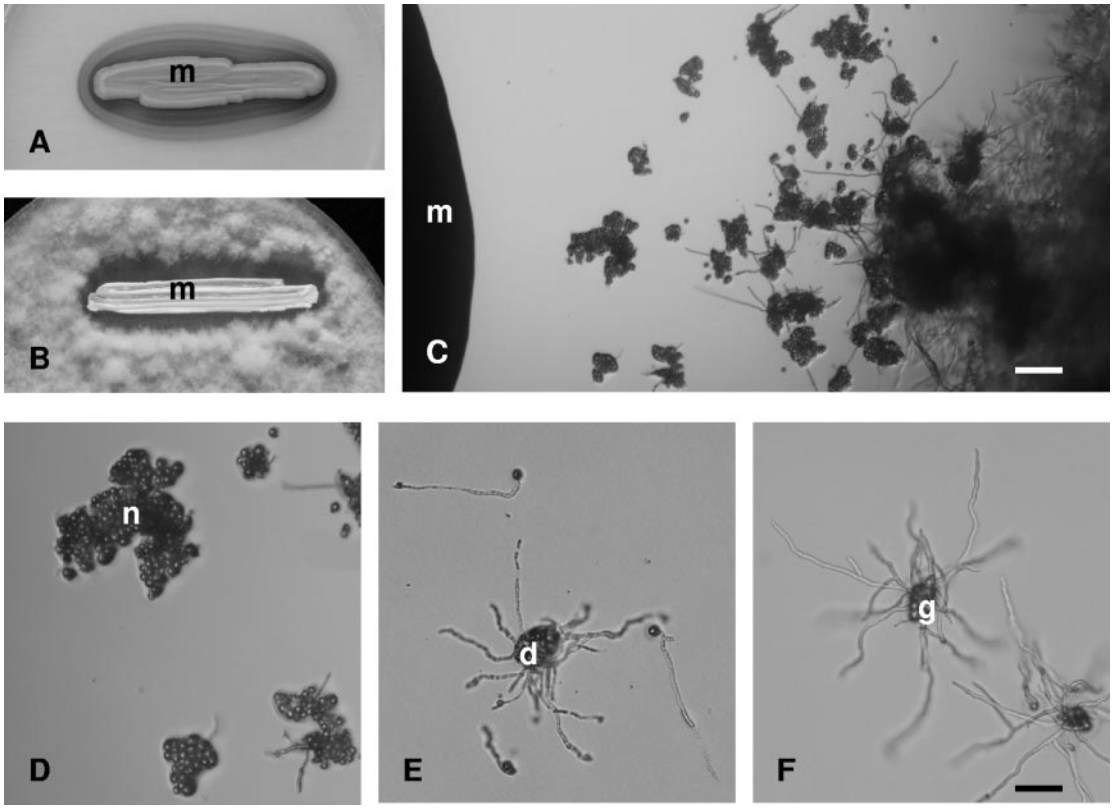

FIG. 1. Halo formation and inhibition of germination of *B. cinerea* conidia by *Metschnikowia* isolate 02.11.1.21. (A) Pigmented halo on YPD supplemented with 0.005 mg/ml $FeCl_3$. (B) Inhibition zone on YPD. (C to F) Microscopic images of conidia on PDA. m, *Metschnikowia* colony; n, group of nongerminating conidia (within a colored halo); d, conidia with dying germination tubes (at the edge of a colored halo); g, group of germinating conidia developing mycelium (outside a colored halo). (C) Bar = 120 μm. (F) Bar = 50 μm.

(10^7 cells/ml) supplemented with 300 μg/ml 1-methyl-3-nitro-1-nitrosoguanidine (catalog no. 12,994-1; Sigma-Aldrich), and incubated in a shaking incubator (100 rpm) at 25°C with daylight (on a laboratory bench in an air-conditioned room). After 30 min, samples were diluted and spread on PDA plates supplemented with 10 μg/ml $FeCl_3$. After 5 days of incubation at 25°C in continuous darkness, colonies that differed from the wild type in colony color or in the size of the pigmented halo were isolated. Colonies with these morphological properties occurred with a frequency of 8×10^{-4}. The survival rate was 23%. The 26S rRNA gene from these colonies was sequenced, and strains that had sequences that were different from that of the mutagenized wild type were discarded. Pigmentation, halo formation, and antifungal activity of the mutants were tested as described above. Because of the lack of hybridization methods for *Metschnikowia*, the mutants were not tested for allelism or to determine the numbers of mutations in their genomes.

Nucleotide sequence accession numbers. Partial 26S rRNA gene sequences of the following strains were deposited in the GenBank database: *Metschnikowia* strains 02.4.3.38 (accession no. DQ666681) and 02.11.1.21(DQ666682) and *B. cinerea* strain 3318 (DQ666677).

RESULTS

Isolation and taxonomic identification of *M. pulcherrima*-like strains. Grape berries were collected from botrytized bunches in two vineyards (Tarcal and Mad) in the Hungarian portion of the Tokaj wine-growing region in 2002 and 2003. Yeasts producing reddish or pink colonies surrounded by reddish halos (Fig. 1A) were recovered from 48/60 berries examined and were more abundant in berries in more advanced stages of noble rotting.

One isolate from each berry was identified to the species level as previously described (49). The carbon and nitrogen source utilization patterns were consistent with identification of these strains as *M. pulcherrima* (see reference 30 for *Metschnikowia* taxonomy). The sequences of the D1/D2 domains of the 26S rRNA gene regions of 10 of these isolates were 96 to 98% identical to those of the type strain of *M. pulcherrima* (accession no. U45736).

Pigment production in cultures of the isolates. Pulcherrimin production is a characteristic of *M. pulcherrima*, but the intensity of pigmentation varies and depends on medium composition (21, 26). The strains isolated in this study varied in color intensity and in the size of the reddish halos that surrounded the colonies (Table 2). The most pigment was produced on YPD, while colonies on PDA and SMA plates either were colorless (white) or turned slightly pink with a barely visible pink halo. Supplementation of the media with $FeCl_3$ enabled pigment production on these media, and the color intensity increased with the iron concentration (Table 2). This finding is consistent with the proposed role of iron in the chemical structure of pulcherrimin (26).

Increasing the concentration of $FeCl_3$ also increased the amount of pigment produced in the liquid media. Without supplementation, YPDL and YEL cultures were pink and SML cultures were colorless. If these cultures were supplemented with 0.1 mg/ml $FeCl_3$ in early stationary phase, they turned red almost instantly, which suggests that the cells pro-

TABLE 2. Formation of pigmented halo and inhibition of the growth of *B. cinerea* around the colonies of selected *Metschnikowia* isolates on PDA plates[a]

Strain	No $FeCl_3$			0.005 mg/ml $FeCl_3$			0.01 mg/ml $FeCl_3$			0.015 mg/ml $FeCl_3$			0.02 mg/ml $FeCl_3$		
	Colony color	Width of colored halo (mm)[b]	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)
02.4.3.38[c]	White	6	7	Pink	2.5	3.5	Red	1.5	1.5	Red	1.5	1.5	Dark red	0.5	0.5
02.11.1.21	White	7	8	Pink	3	3	Red	1.5	1.5	Red	0.5	0.5	Dark red	0	0
7.3.37	White	7	8	Pink	2	3	Red	1	1	Red	0.5	0.5	Dark red	0	0
17.1.IV	Pink	5.5	6	Pink	2	3.5	Red	2	2	Red	0.5	0.5	Dark red	0	0
17.3.1.	White	7	8	Pink	3	4	Red	2	2	Red	1.0	1.0	Dark red	0.5	0.5

[a] Results were determined on the 10th day of incubation. Data for four experiments are shown. The widths of the halos and inhibition zones were measured manually. Due to the identical conditions and because of the resolution limit of manual measurement, no detectable deviations were observed.
[b] Faintly visible.
[c] Strains 4a.3.11, 7.3.10, 9.4.60, and 11.3.38 give similar but somewhat weaker reactions.

duce a colorless precursor that is converted to pigment as soon as iron is available. If these red cultures were centrifuged, then both the sediment, consisting of yeast cells, and the cell-free supernatant were red. If 0.1-ml samples of the supernatants were placed into wells cut into PDA and SMA plates, no pigment halos appeared around the wells. Presumably, the pigment could not diffuse into the medium, perhaps because it was suspended rather than dissolved in the sample. With time, the pigment slowly precipitated to form a maroon sediment if the supernatant was not agitated. The precipitated material did not redissolve in water.

If the pigment is insoluble in water and cannot diffuse into the medium, then it must be formed in situ from its soluble precursors. Pulcherrimin is formed from leucine through the intermediates cyclo-L-leucyl-L-leucyl and pulcherriminic acid (29). Since all of these compounds are water soluble, any of them could be the secreted precursor from which the pigmented halo is formed around the colonies. To test the possibility that leucine is the secreted precursor, the most active *Metschnikowia* isolates were streaked on SMA plates seeded with cells of the leucine-dependent strain *Schizosaccharomyces pombe leu1*. No cross-feeding of the *Schizosaccharomyces* mutant was observed around the *Metschnikowia* colonies, indicating that the *Metschnikowia* cells did not excrete leucine and that the secreted precursor of pulcherrimin is not leucine.

Inhibition of germination of *Botrytis* conidia. Fifteen colonies with *Botrytis* morphology were isolated, and their D1/D2 rRNA gene domains were sequenced, which confirmed their identification as *B. cinerea* (100% identity with the accession no. AF250919 sequence, the sequence of a *B. cinerea* [*Botryotinia fuckeliana*] strain isolated from Californian grapes). One of these isolates, strain 3318, was used as a tester (GenBank accession no. DQ666677). The other tester was strain 980, a strain isolated from grapes in France (22).

All *Metschnikowia* isolates inhibited germination of the conidia of both *B. cinerea* testers (Fig. 1C and D) if the spores were within 0 to 7 mm (depending on the strain and the amount of $FeCl_3$ added to the medium) of the *Metschnikowia* colony. At 0 to 6.5 mm from the *Metschnikowia* colony (depending on the strain and the amount of $FeCl_3$ added to the medium), the conidia germinated but died after forming short germination tubes (Fig. 1E), suggesting that the presence of the *Metschnikowia* isolates could inhibit hyphal growth. The distance across which the *Metschnikowia* isolates caused this antagonistic effect varied by strain. Strains that produced larger halos usually also had larger inhibition zones (Table 2). Outside the inhibition zones, the conidia germinated uniformly and formed dense mycelium (Fig. 1B and F). After 5 to 6 days of incubation, the mycelium began to grow slowly into the inhibition zone and gradually reduced the size of the zone. No differences were detected between the two *Botrytis* tester strains.

Inhibition of growth of *Botrytis* hyphae. The *Metschnikowia* isolates also were inoculated ahead of the growing front of the *Botrytis* mycelium. After a few hours of incubation, mycelia near the yeast colony stopped growing and the hyphal morphology changed (Fig. 2A). Many hyphae lysed at the tip, and in others extensive protoplasmic coagulation occurred. Forty-

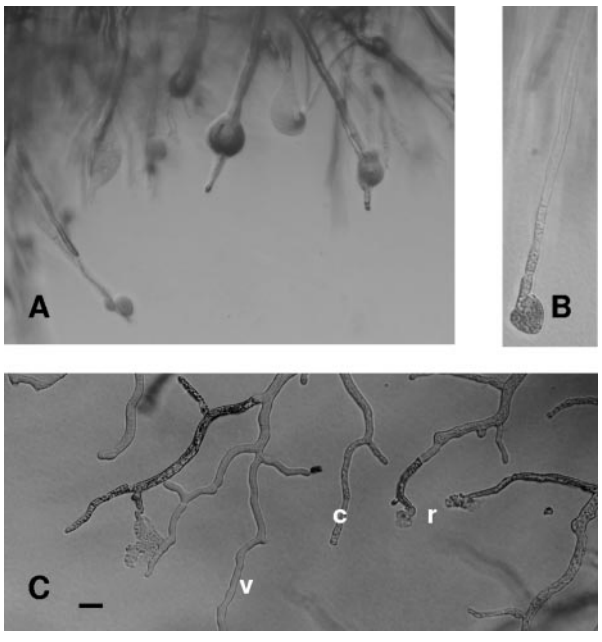

FIG. 2. Degeneration of hyphae at the edge of the inhibition zone around a colony of *Metschnikowia* isolate 02.11.1.21. (A) *B. cinerea* 3318. (B) *A. pullulans* 27/2.36. (C) *M. piriformis*. v, viable hypha; r, hypha ruptured near its tip; c, hypha with coagulated cytoplasm. Bar = 20 μm.

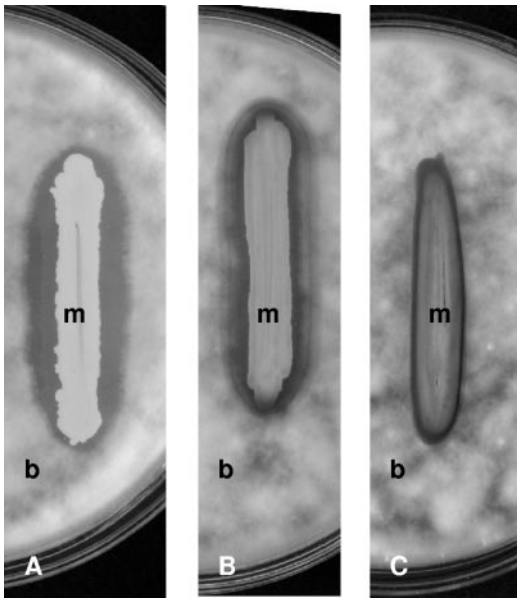

FIG. 3. Effect of $FeCl_3$ on halo formation and inhibition of the growth of *B. cinerea* on PDA. (A) Without $FeCl_3$. (B) With 0.005 mg/ml $FeCl_3$. (C) With 0.02 mg/ml $FeCl_3$. m, *Metschnikowia* colony; b, *Botrytis* mycelium.

eight to 72 h later some hyphae resumed growing and grew slowly toward the yeast colony.

Iron and antifungal antagonism. Five *Metschnikowia* isolates were inoculated onto PDA plates supplemented with various amounts of $FeCl_3$ and then flooded with *Botrytis* conidia. The greater the amount of iron added, the narrower the halos and inhibition zones were (Table 2). At exogenous $FeCl_3$ concentrations greater than 20 μg/ml no red halos appeared, and the growth of *Botrytis* was not inhibited (Fig. 3). Increasing the iron concentration reduced the size of the halos but increased the pigmentation of the yeast colonies. At $FeCl_3$ concentrations greater than 20 μg/ml the yeast colonies were dark red, suggesting that all of the pigment was retained in the cells. The simultaneous reductions in the sizes of the pigment halos and inhibition zones by iron supplementation suggests that the *Metschnikowia* isolates inhibited *B. cinerea* by depleting the iron in the medium.

Tropolone is a chelating agent with a strong affinity for ferric ions (12, 17). Tropolone was placed into holes (0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 mg/hole) in PDA plates seeded with *Botrytis* conidia, which resulted in growth inhibition similar to that observed with the *Metschnikowia* isolates (Fig. 4). This effect could be reversed by supplementing the medium with $FeCl_3$, demonstrating that iron depletion inhibits conidial germination. The *Botrytis* testers each formed a ring of thick mycelium around the inhibition zone (Fig. 4), which could be attributed to nutrient diffusion from the inhibition zone. As observed with the *Metschnikowia*-generated inhibition, hyphae began growing slowly toward the wells after 4 to 5 days of incubation. Tropolone also inhibited the formation of the red or maroon pigment. When 1.0 mg tropolone was placed into a hole cut into the medium (15 μg/ml $FeCl_3$) 10 mm from a *Metschnikowia* colony, the colony did not turn red and did not form a halo in the agar.

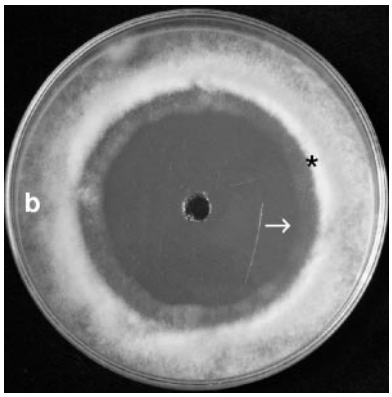

FIG. 4. Inhibition of *B. cinerea* by tropolone. Fifty microliters of a solution containing 0.5 mg tropolone was placed into a hole in the middle of the plate. The asterisk indicates the ring of stimulated mycelial growth. The arrow indicates hyphae growing into the inhibition zone. b, *Botrytis* mycelium.

Correlation between pigment production and antifungal activity in mutants. Cells of isolates 02.11.1.21 and 02.4.3.38 were mutagenized with nitrosoguanidine, and mutant colonies that differed from the wild-type colonies in pigmentation were recovered. Three white (colorless) mutants, eight pink mutants (with various degrees of pigmentation), and one mutant whose colonies formed halos that were broader than the halos of the wild-type colonies were selected for further examination. The mutants were tested for pigment production and the ability to inhibit germination of *B. cinerea* conidia on PDA supplemented with various amounts of $FeCl_3$. The colonies formed by colorless mutants A-1, A-2, and G-21 were white at all concentrations of iron and did not affect the growth of *Botrytis*. Mutant G-38 was more pigmented and had a wider halo and stronger inhibitory effect on *Botrytis* than the wild-type parent. The other mutants had lower, but not uniform, amounts of pigmentation and antifungal antagonism (Table 3). The white and low-pigment mutants grew as well as the wild-type strains at all concentrations of iron tested (0 to 0.5 mg/ml) at pH 5.0 and 6.5. These conditions did not inhibit the growth of *Botrytis*.

Activity against other microorganisms. The *Metschnikowia* strains also were tested for activity against other fungal species (Table 1). Most of these fungi were as sensitive to the *Metschnikowia* strains as *B. cinerea* was (Table 4). *Aspergillus niger* conidial germination was not affected, but mycelial growth was retarded. Massive lysis of hyphae occurred in *A. pullulans* and *Mucor piriformis* (Fig. 2B and C).

The environment from which the *Metschnikowia* strains were isolated is rich in yeasts and bacteria (1, 3, 45). Therefore, I also tested the *Metschnikowia* isolates for activity against yeasts and bacteria. The growth of *Candida stellata*, *Hanseniaspora uvarum*, *S. pombe*, and *Schizosaccharomyces japonicus* was not altered by the presence of *Metschnikowia*. The growth of *Saccharomyces cerevisiae* and *Saccharomyces uvarum* was somewhat retarded around the *Metschnikowia* colonies, but no inhibition zones with sharp edges were formed (Fig. 5A). A similar reaction was described by Nguyen and Panon (34). *Candida zemplinina* also was sensitive; its growth was severely inhibited near the *Metschnikowia* colonies, but thin rings of facilitated growth fringed the inhibition zones (Fig. 5B).

TABLE 3. Production of pigmented halos and the inhibition of *B. cinerea* growth around colonies of wild-type *Metschnikowia* isolates and mutants on PDA plates[a]

Strain	No $FeCl_3$			0.01 mg/ml $FeCl_3$			0.015 mg/ml $FeCl_3$		
	Colony color[b]	Width of colored halo (mm)[c]	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)	Colony color	Width of colored halo (mm)	Width of inhibition zone (mm)
Wild-type strains									
02.4.3.38	FP	4.5	4	R	1.0	1	DR	0.5	0
02.11.1.21	FP	4	4	R	1.5	1	DR	0.5	0
Mutant strains isolated from 02.4.3.38									
G-38	FP	6	4	P	4	2	R	3.5	2
H-1	FP	4	2.5	R	0	0	R	0	0
H-2	FP	3.5	2.5	R	0.5	0	R	0.5	0
Mutant strains isolated from 02.11.1.21									
A-1	W	0	0	W	0	0	W	0	0
A-2	W	0	0	W	0	0	W	0	0
B-1	W	0	0	FP	0	0	FP	0	0
B-2	W	0	0	FP	0	0	FP	0	0
C	W	0	0	P	0.5	0	R	0	0
D	FP	4	2	P	0.5	0	R	1	0.5
E	W	0	0.5	P	0.5	0	R	0	0
F	FP	4	3	P	2.5	1.5	R	1	0.5
G-21	W	0	0	W	0	0	W	0	0

[a] Results were determined after 6 days of incubation. Data for four experiments are shown. The widths of the halos and inhibition zones were measured manually. Due to the identical conditions and because of the resolution limit of manual measurement, no detectable deviations were observed.
[b] W, white (colorless); FP, faint pink; P, pink; R, red; DR, dark red.
[c] Faintly visible.

O. oeni did not grow close to a *Metschnikowia* colony but did form a broad halo of vigorous growth around the very thin inhibition zone. *E. coli* was more sensitive to *Metschnikowia*; it was inhibited more severely and did not grow beyond the zone of inhibition.

DISCUSSION

The red or maroon pigment (pulcherrimin) of *M. pulcherrima* has been the subject of numerous previous studies. This pigment forms reddish halos around colonies of *M. pulcherrima*. These pigmented halos contain a water-insoluble complex of ferric ions and pulcherriminic acid (11, 26). The reddish brown colonies of the *Metschnikowia* isolates analyzed in this study also were surrounded by colored halos, and their pigment also was insoluble in water.

The reduction in halo size observed when the medium was supplemented with $FeCl_3$ suggests that the cells do not secrete the pigment but instead secrete a soluble, diffusible precursor that forms the pigment in the medium when it encounters iron. At low iron concentrations the precursor diffuses further from the yeast colony before it is immobilized by iron, resulting in a wider but paler halo. At higher iron concentrations the halos are smaller because the precursor molecules do not diffuse as far before they bind sufficient iron for pigment production. Since the pigment in these cases is concentrated in a smaller area, the color of the resulting halos is more intense. At even higher concentrations of iron, no halo is produced, suggesting that the entire precursor pool is converted to pigment within the cells. At these concentrations the yeast colonies are dark red (maroon).

The pigment, pulcherrimin, is synthesized from L-leucine through cyclo-L-leucyl-L-leucyl and pulcherriminic acid (29). Since the *Metschnikowia* colonies do not cross-feed yeast leucine auxotrophs, leucine can be ruled out as the secreted precursor. Chemical analysis of the medium is needed to determine which of the other two intermediates is released.

As indicated by Kluyver et al. (26), Beijerinck hypothesized that pigment production is a defense reaction of the *M. pulcherrima* cells against the presence of excess iron. However, both Beijerinck and Kluyver et al. (26) described colorless colonies in their *M.* (*Torula*, *Candida*) *pulcherrima* cultures, which argues against such a role. If the organism does need protection against iron, then the inability to form pigment should be selected against. The pigmentless mutants isolated in this study grew like the wild type even at iron concentrations that turned the wild-type colonies dark red. Thus, pigment production is not essential for growth, even under high-iron conditions, so a protection function is unlikely and pigment production must benefit the organism in some other manner.

The antimicrobial activity of the isolates suggests that a biological function for pigment production might be to inhibit the growth of other microbes that are potential competitors for nutrients. The pulcherrimic acid-ferric ion complex formed in the halos depletes the iron in the substrate and creates an environment unsuitable for growth of microbes that require more iron for growth. This hypothesis is based on the observed correlation between the size of the pigmented halos around the *Metschnikowia* colonies and the size of the zones within which the growth of *Botrytis* was inhibited. *Metschnikowia* isolates that produced wider halos also had a stronger antagonistic

TABLE 4. Antagonistic effects of *Metschnikowia* against various microorganisms

Strain	Width of inhibition zone (mm) around colonies of *Metschnikowia* strain[a]:		Remarks
	02.4.3.38	02.11.1.21	
Aspergillus clavatus IMI 15949	2	2.5	
Aspergillus niger IMI 17454	0	0	Growth reduction
Aureobasidium pullulans 27/2.36	1	0	Growth reduction with no sharp edge; dark band around the *Metschnikowia* colony[b]
Botrytis cinerea 980	3.5	4	Growth stimulation zone, 1 mm
Botrytis cinerea 3318	4	4	Growth stimulation zone, 1 mm
10-432 *Candida stellata*	0	0	
10-372 *Candida zemplinina*	3	5	Growth stimulation zones, 3 mm for 02.4.3.38 and 0.5 mm for 02.11.1.21[c]
Escherichia coli DH5	2	2.5	
Gilbertella persicaria IMI 101638	3.5	4.5	
10-511 *Hanseniaspora uvarum*	0	0	
Mucor piriformis NRRL 3636	2	2	
Mucor circinelloides ATCC 1216	2	2	
Oenococcus oeni B16	0	0.5	Growth stimulation zone, 3 mm
Rhizopus stolonifer var. *stolonifer* CBS 109.76	4.5	5.5	
Saccharomyces cerevisiae S288c	0	0	Growth reduction with no sharp edge[d]
10-408 *Saccharomyces uvarum*	0	0	Growth reduction with no sharp edge
7-1 *Schizosaccharomyces japonicus* var. *japonicus* CCY-44-5-1	0	0	
0-1 *Schizosaccharomyces pombe* var. *pombe* L972	0	0	

[a] Results were determined after 6 days of incubation. Data for four experiments are shown. The widths of the halos and inhibition zones were measured manually. Due to the identical conditions and because of the resolution limit of manual measurement, no detectable deviations were observed.
[b] See Fig. 5C.
[c] See Fig. 5B.
[d] See Fig. 5A.

effect on *B. cinerea*. The mutants that lacked pigment did not affect *Botrytis*, and strains producing low levels of pigment also had lower levels of antifungal activity. Any reduction in halo size due to supplementation of the medium with iron also reduced the size of the inhibition zone.

An alternative hypothesis is that either the pigment or its precursors have antimicrobial activity and that iron depletion is an incidental and harmless side effect. I cannot exclude this possibility as I have not tested purified pigment or its precursors for this activity; however, the iron-binding agent tropolone (17) also inhibits the germination of conidia and the growth of the test organisms. Iron also may be involved in plant antifungal defense mechanisms since infection by *B. cinerea* increased the Fe(III) levels in tissues of *Capsicum annuum* and *Arabidopsis thaliana* (15, 32).

Sequestration of iron is a widespread mechanism of antimicrobial antagonism. Siderophores (low-molecular-weight, ferric ion-specific chelating agents) that deplete the iron in the environment by transporting it into the cells of the siderophore-excreting microorganisms can inhibit the growth of other microbes that do not have similar mechanisms (6, 23, 24, 33). The ferrisiderophore complexes are water soluble, which makes them accessible to the specific membrane receptor and transport systems that mediate their transport into the cell (for a review, see reference 51). The pigment produced by the *Metschnikowia* cultures also might act as a siderophore, but the results of this study make this possibility unlikely. The *Metschnikowia* pigment is not soluble in water and cannot diffuse in the agar medium, so rather than solubilizing the iron in the environment, it immobilizes it. Nevertheless, low-molecular-weight forms of the pigment complex might diffuse to the cell and deliver ferric ions.

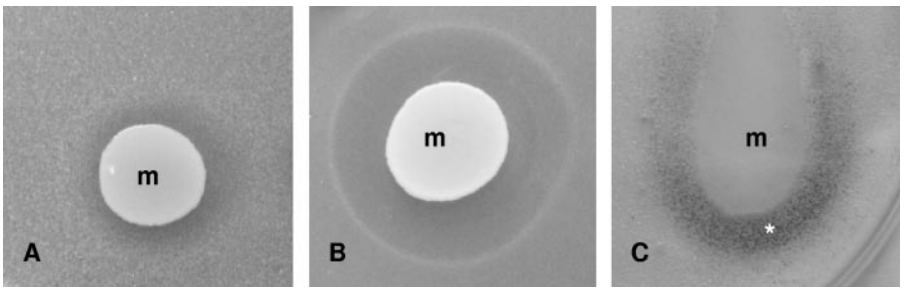

FIG. 5. Effect of *Metschnikowia* isolate 02.11.1.21 on yeasts. (A) *S. cerevisiae*. (B) *C. zemplinina*. (C) *A. pullulans* (yeast phase). m, *Metschnikowia* colony. There is a zone (marked with an asterisk) in which there is increased melanin production in the *A. pullulans* lawn.

The accumulation of the pigment within a *Metschnikowia* colony at higher iron concentrations also argues against a siderophore function. Microscopic observations made by Kluyver et al. (26) indicate that in highly pigmented colonies the pigment is encrusted on the cell wall and partially covers the outside of the cell. Siderophore-iron complexes do not accumulate in the cell but instead dissociate to free the iron for cellular metabolism (13). Another difference between the *Metschnikowia* pigment and siderophores is in the regulation of their production. Siderophores usually are produced under iron-limiting conditions (for a review, see reference 24), whereas the *Metschnikowia* pigment is formed constitutively, at both low and high ferric ion concentrations.

Based on microscopic observations of the test organisms, the iron-limited environment created by a *Metschnikowia* colony is lethal to germinating conidia. This finding is consistent with the results of Charlang et al. (8), who found that the conidia of *Aspergillus nidulans* and *Penicillium chrysogenum* required intake of a large amount of iron for germination. More recently, rhodotorulic acid, a siderophore produced by *Rhodotorula glutinis*, was shown to reduce conidial germination of *Penicillium expansum* (6) and *B. cinerea* (43). However, there have been no studies of the cytological effects of iron limitation on mycelium. The observation that hyphae crack when they enter the pigmented zones that form around the *Metschnikowia* colonies is a new finding and demonstrates that iron starvation elicits complex physiological changes in the fungal cells.

Interestingly, the inhibition of hyphal growth is transitory, and after a lag period of a few days the mycelium recovers from the shock and begins to grow slowly into the inhibition zone. The pigment may degrade with time (and release iron), or iron may be supplied to the growing hyphal tips by cytoplasmic transport from pigment-free areas of the medium. Since recovery also was observed at the edges of the tropolone-generated inhibition zones, the latter possibility is more likely. Numerous fungi can transport nutrients, including iron, between various parts of the mycelium (5, 17).

Some test organisms (*O. oeni*, *B. cinerea*, and *C. zemplinina*) formed rings of thicker growth around the inhibition zones. The mechanism of this stimulation is not known, but the stimulation may be attributable to the diffusion of nutrients from the inhibition zones. For example, for *O. oeni*, a bacterium that is common on grapes and in wines (28), the ring of facilitated growth was much wider than the inhibition zone. The polymorphic organism *A. pullulans* also responded to the presence of *Metschnikowia* in two ways. In addition to growth inhibition, the presence of *Metschnikowia* also elicited melanin production by the yeast-like cells, a process characteristic of chlamydospores (39).

In summary, *Metschnikowia* yeasts growing on noble-rotted grapes can strongly antagonize the growth of various filamentous fungi, yeasts, and bacteria by producing an insoluble pigment that depletes the iron in the environment. The immobilization of iron rather than incorporation of iron into the antagonizing strain is a novel method of extracting iron from the environment for biological control. Such a mechanism has not been demonstrated previously and may provide a new strategy for use in the biological control of various plant pathogens.

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