Isolation and Characterization of New *Metschnikowia pulcherrima* Strains as Producers of the Antimicrobial Pigment Pulcherrimin

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Z. Naturforsch. **64c**, 405–410 (2009); received October 31, 2008/January 8, 2009

Metschnikowia pulcherrima is a highly effective biocontrol yeast due to its pigment pulcherrimin that accumulates in the cells and in the growth medium. Three different strains of *M. pulcherrima* were isolated from local grapes. The yeast isolates were characterized on the basis of their biochemical, physiological and ITS1-5.8 s rDNA-ITS2 region. Based on the obtained results, the *M. pulcherrima* isolates were identified as new strains of *M. pulcherrima*. Strong antagonistic activities of the *M. pulcherrima* strains on the human pathogens *Proteus vulgaris*, *Escherichia coli*, *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, and *Trichosporon mucoides* were determined. In addition, antagonistic effects of these *M. pulcherrima* strains were also tested against *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma* spp., *Paecilomyces* spp., and *Bipolaris* spp. and it was shown that the three different strains of *M. pulcherrima* also have an antagonistic effect on the growth of these fungal species at different extents. This study showed that all three strains of *M. pulcherrima* produce the same amount of the pigment pulcherrimin, but their antimicrobial activities on different microorganisms show important variations.

Key words: Biocontrol, Pulcherrimin, Antagonistic Yeasts

Introduction

The large-scale use of chemicals to prevent microbial diseases in agriculture often results in heavy contamination of lands and water resources. It is well known that bacteria or even yeasts can develop resistance to antibiotics and fungicidal chemicals (Espinel-Ingroff, 2008). Biological control of microbial infections has a great potential as an alternative approach to chemical-based methods. One of the promising methods is the use of antagonistic microorganisms to prevent growth and infections of unwanted microbes. Antagonistic effects of yeasts, used as biocontrol agents, are mostly based on the competition for nutrients and growth space (Droby and Chalutz, 1994).

Strains of *Metschnikowia pulcherrima* have a great potential as biocontrol yeasts against postharvest pathogens (Piano *et al*., 1997; Qin *et al*., 2004; Spadaro *et al*., 2002). The natural habitats of *M. pulcherrima* strains are fully matured fruits, especially grapes (Mills *et al*., 2002). *M. pulcherrima* produces pulcherrimin, which is a red pigment that accumulates in the growth medium and, clearly visible, around the *M. pulcherrima* colonies on plates (Kluyver *et al*., 1953). Pulcherrimin forms a chelate complex with iron ions in the medium (Kluyver *et al*., 1953). It has been recently shown that the antibacterial and antifungal activity of *M. pulcherrima* strains depends on the immobilization of iron by the pulcherrimin pigment in the growth medium (Sipiczki, 2006). Hence, the strains of *M. pulcherrima* that produce high amounts of pulcherrimin are of great interest for the inhibition of the growth of pathogenic bacteria, yeasts, and molds.

Antagonistic effects of *M. pulcherrima* strains on plant pathogens were identified previously (Sipiczki, 2006). The aim of the present study was to isolate new *M. pulcherrima* strains from local habitats and to test their antagonistic activities against human pathogens that lead to bacterial or fungal infections.

Material and Methods

Isolation of M. pulcherrima strains

Berries of black grape (local variety of *Vitis vinifera* L. ssp. *silvestris* Gmel.) were collected aseptically from the local vineyards in Düzce

0939–5075/2009/0500–0405 $ 06.00

province, Turkey in September 2007. 10 g of berries were mildly homogenized in 100 mL of sterile distilled water. 100 μL from the resulting mixture were spreaded on YGC agar (5 g/L yeast extract, 20 g/L glucose, 0.1 g/L chloramphenicol, 14.9 g/L agar) plates supplemented with 0.1% sodium propionate and incubated at 30 ºC for 3 d. 100 colonies were randomly selected for species identifications. Yeast strains were cultivated in YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) medium for further analysis. Species identification of the isolated yeasts was done with the API ID32c yeast identification system (Bio Merieux, Lyon, France) following the manufacturer's suggestions.

Three different strains of *M. pulcherrima* (UMY12, UMY14, and UMY15) that give reddish halos on the YPD plates were selected randomly for further studies. In addition to API ID32c tests, species identifications of these *M. pulcherrima* strains were also done by analysis of ITS1-5.8 s rDNA-ITS2 regions of the isolated yeast species (White *et al*., 1990). Genomic DNA was isolated from the *M. pulcherrima* strains as described previously (Sherman *et al*., 1986). ITS1-5.8 s rDNA-ITS2 regions were amplified with ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. The polymerase chain reaction (PCR) conditions were same as described by White *et al*. (1990). 3 μL-Aliquots of the amplified products were separated on 1.5% (w/v) agarose gel, then stained with SYBR-Green and photographed under UV light with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA) gel documentation system. To determine the sequence of the ITS regions of the *M. pulcherrima* strains, resulting PCR fragments were purified and sequenced with DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), and then analyzed with ABI PRISM 310 Genetic Analyzer (Iontek, Istanbul, Turkey). Sequence similarity analyses were done using the BLAST service of the NCBI. The nucleotide sequences of the ITS1-5.8 s rDNA-ITS2 regions of the *M. pulcherrima* strains determined in the present study have been deposited in GenBank and given the following accession numbers: FJ172528 (strain UMY12), FJ172527 (strain UMY14), and FJ172526 (strain UMY15).

Microorganisms

The microorganisms were revitalized from the stocks in our laboratories. The microorganisms used in the antagonistic activity tests were: *Escherichia coli* DH5α, *Proteus vulgaris* ATCC 13315, *Saccharomyces cerevisiae* H251, *Debaryomyces occidentalis* DBVPG-6722, *Kluyveromyces marxianus* CBS 4857, *Candida albicans* ATCC 24433, *Candida albicans* ATCC 10231, *Candida albicans* CBS 2730, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Candida dupliniensis* CD 36, *Candida tropicalis* ATCC 1021, *Trichosporon mucoides* H 295, *Aspergillus niger* ATCC 16604, *Aspergillus flavus* ATCC 22293, *Aspergillus terreus* 22535, *Aspergillus fumigatus* ATCC 22626, *Paecilomyces* spp., *Mucor* spp., *Trichoderma* spp., and *Bipolaris* spp.

Antagonistic activity tests

The isolated *M. pulcherrima* strains UMY12, UMY14, and UMY15 were grown in YPD medium until the logarithmic stages (OD_{600}:1) in an incubator shaker with 130 rev/min at 30 ºC (Sherman *et al*., 1986). *E. coli* DH5α and *Proteus vulgaris* were grown in 10 mL nutrient broth medium (Merck, Darmstadt, Germany) at 37 ºC in an incubator shaker at 130 rev/min until the logarithmic stage. Bacterial cultures were diluted 100-fold and then 100 μL of diluted bacterial cultures were spread on nutrient agar plates supplemented with 2% glucose. When the surface of the bacterial plates dried, 4 μL of *M. pulcherrima* samples were dropped on each plate in duplicate (Sipiczki, 2006). Plates were incubated at 37 ºC for 2 d and the inhibition zones were measured manually. Inhibition zones were defined as the distance extending from the edges of the *M. pulcherrima* colonies to the beginning of the bacteria or yeast lawns on the plates and expressed in mm (Fig. 2).

To test the antagonistic activities of the *M. pulcherrma* strains UMY12, UMY14, and UMY15 on the different yeast species, yeast strains were grown in 10 mL of YPD medium up to the logarithmic stage and then diluted 100-fold with distilled sterile water. 100 μL of diluted yeast samples were spread on YNB glucose plates (Sherman *et al*., 1986). After the yeast lawn on each plate was dried, 4 μL of *M. pulcherrima* samples were dropped on each plate in duplicate. The yeast

plates were incubated at 30 ºC for 2 d and the inhibition zones were measured.

Antagonistic effects of the *M. pulcherrima* strains UMY12, UMY14, and UMY15 on spore germination and growth of different mold species were also tested as follows. A loop full of spores from fully sporulated strains of *Aspergillus*, *Mucor*, *Trichoderma*, *Paecilomyces* and *Bipolaris* were collected aseptically and resuspended in 1 mL of sterile distilled water. Then 100 μL of spore suspension (approximately 10^4 spores/plate) were spread evenly on the YNB glucose plates. When the surface of plates dried, 4 μL of *M. pulcherrima* samples were dropped on the plates in duplicate, and the plates were incubated at 30 ºC for spore germination and hypeal growth for 2 d. Inhibition zones were measured at the end of the incubation periods. All experiments on the antagonistic effects of *M. pulcherrima* strains were repeated at least twice. The numbers of the inhibition zones given in the tables are the mean values of at least 4 independent experiments. Since the measurements were done manually, there were no detectable deviations in the inhibition zones among the individual measurements belonging to the same group of microorganisms. The standard deviations for the inhibition zones were less than 0.5 mm.

Results and Discussion

Isolation and identification of M. pulcherrima strains

The grape must samples, which were obtained from 10 g of local black grapes, were screened for the isolation of different *M. pulcherrima* strains. The yeast colonies were purified and classified according to Kurtzman and Fell (2000). The selected colonies were further characterized for their taxonomic status using the API ID32c system. The most abundant yeast species in the grape must samples were; *Pichia norvegensis* (56%), *M. pulcherrima* (18%), *Kloeckera japonica* (13%), and *Kloeckera apiculata* (13%). *M. pulcherrima* and *K. apiculate* were reported previously as two of the three main yeast species in the fermentation medium of grape juice (Combina *et al.*, 2005). Three of the red pigment-producing yeasts on the plates were further characterized for their exact species names. These yeast strains were identified with the API ID32c system as *M. pulcherrima* with 99% accuracy.

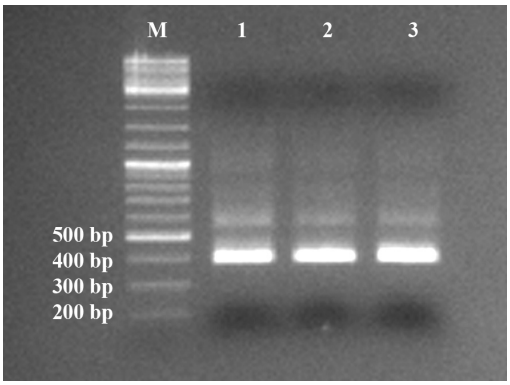

Fig. 1. Agarose gel electrophoresis of ITS1-5.8 s rDNA-ITS2 regions of *M. pulcherrima* strains. Lanes 1–3, PCR amplicons obtained from the genomic DNAs of the *M. pulcherrima* strains UMY12, UMY14, and UMY15, respectively; lane M, DNA molecular marker (Fermentas GeneRuler DNA ladder mix, SM0333). DNA sizes are given as base pairs (bp).

Genomic DNA was purified from these *M. pulcherrima* strains to amplify the ITS1-5.8 s rDNA-ITS2 region. The amplification of these ITS regions by PCR produced approximately 400-bp-long DNA bands (Fig. 1). The sizes of the ITS regions of *M. pulcherrima* strains isolated in the present study were very close to the previously isolated *M. pulcherrima* strain's ITS region (Clemente-Jimenez *et al.*, 2004; Nisiotou and Nychas, 2007). BLAST analysis of the sequence of the ITS1-5.8 s rDNA-ITS2 region also identified these three yeast species as *M. pulcherrima* strains with 93–94% similarity to previously isolated MECH1, BIO126, ZY6, MB513, and MB510 strains of *M. pulcherrima*. Comparison of these ITS-rDNA regions of the isolated *M. pulcherrima* strains (UMY12, UMY14, and UMY15, respectively) showed that they are highly homologous to each other with 98% identity. In addition, their pulcherrimin pigment production efficacy seemed to be the same as shown by the zones of red pigment measured on the plates (Gimenez-Jurado *et al.*, 1995; Sipiczki, 2006).

Antagonistic activities of M. pulcherrima strains on bacteria and yeasts

The inhibitory effects of pulcherrimin, that is synthesized by *M. pulcherrima* strains, on different microorganisms were tested by plate assays. We have found that although *M. pulcherrima*

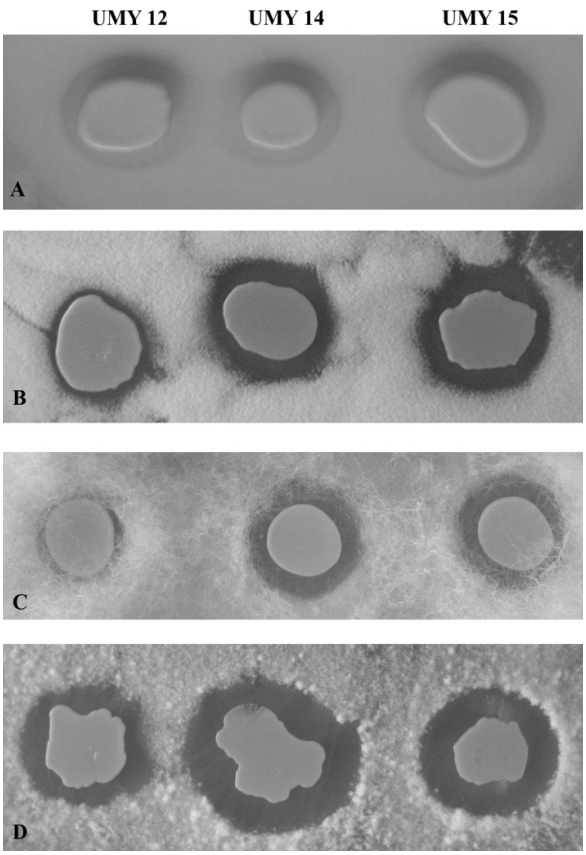

Fig. 2. Antagonistic effects of the *M. pulcherrima* strains UMY12, UMY14, and UMY15 on different microorganisms. Samples from the liquid cultures of the *M. pulcherrima* strains UMY12, UMY14, and UMY15 were dropped on the freshly plated lawns of the following microorganisms: (A) *Proteus vulgaris*; (B) *Paecilomyces* spp.; (C) *Mucor* spp.; (D) *Trichoderma* spp.

strains isolated from local grapes produced the same levels of pulcherrimin, they showed variations in their antagonistic effects against bacteria, yeasts, and mold species tested in this research. *M. pulcherrima* strain UMY12 had the strongest antagonistic effect both on *E. coli* and also on *Proteus vulgaris*. While *M. pulcherrima* strains UMY14 and UMY15 had a strong inhibitory effect on *Proteus vulgaris*, they had no effect on the growth of *E. coli* DH5α cells (Table I, Fig. 2A). The differences in the antagonistic effects of *M. pulcherrima* strains UMY12, UMY14, and UMY15 were also apparent on the yeasts. While the *M. pulcherrima* strain UMY15 had a strong antagonistic activity on most of the yeasts, strain UMY12 had a weak or no antagonistic effect on most of the yeast species tested (Table I). This difference was very clear in their effects on the growth of the various strains of *C. albicans*. Differential antagonistic effects of *M. pulcherrima* strains on the *Aspergillus* and *Penicillium* species were reported previously (Janisiewicz *et al.*, 2001; Bleve *et al.*, 2006).

All three strains of *M. pulcherrima* showed a high degree of identity with respect to their rDNA region, and they all produced the same level of red pulcherrimin pigment. Hence, the reason for their differential antagonistic effects might depend on other aspects of the pigment pulcherrimin. It is known that pulcherriminic acid, the iron-free form of pulcherrimin, is present in different tautomeric forms in different *M. pulcherrima* strains (MacDonald, 1963). Hence it is conceivable that the efficacy of iron immobilization by pulcherrimin produced from the three strains might be different. It is known that iron is one of the essential elements for most of the microorganisms (Howard, 1999). As a result, immobilization of iron in the growth medium by pulcherrimin results in inhibition of the microbial growth on test plates.

It is known that the presence of high amounts of iron in the growth medium eliminates the antagonistic effects of pulcherrimin (Sipiczki, 2006). We have also tested the effect of excess iron (0.02 μg/mL $FeCl_3$) on the antagonistic effects of *M. pulcherrima* strains and found that the presence of excess iron in the YNB glucose plates completely eliminates the antagonistic effects of *M. pulcherrima* strains (data not shown). In addition, when the pH value of the growth medium increased to 7.4, there was no inhibitory effect of the *M. pulcherrima* strains against the microorganisms tested. It seems that the pigment pulcherrimin does not bind iron at this pH value.

Antagonistic effects of M. pulcherrima strains on spore germination

M. pulcherrima strains also have a very strong antagonistic activity on spore germination of various fungal species. In this case, the *M. pulcherrima* strains UMY14 and UMY15 had strong antagonistic activities on the germination of *Trichoderma* and *Aspergillus* spores. *M. pulcherrima* strain UMY12 had a moderate to low level of antagonistic effect on spore germination in all

Table I. Antagonistic effects of *M. pulcherrima* strains on various bacteria and yeasts.

Microorganism	Inhibition zone[a] [mm]		
	UMY12	UMY14	UMY15
Escherichia coli	4	NZ	NZ
Proteus vulgaris	5	4	4
Saccharomyces cerevisiae	2	3	3
Debaryomyces occidentalis	3	4	4
Kluyveromyces marxianus	NZ	2	5
Candida albicans 24433	1	1	3
Candida albicans 10231	NZ	2	3
Candida albicans 2730	NZ	1	3
Candida parapsilosis	2	4	5
Candida krusei	1	3	4
Candida dupliniensis	1	2	2
Candida tropicalis	NZ	2	2
Trichosporon mucoides	1	4	4

[a] Inhibition zones were measured from the edges of *M. pulcherrima* colonies to the beginning of the bacteria or yeast lawns.
NZ, no zone of inhibition.

Table II. Antagonistic effects of *M. pulcherrima* strains on different fungal species.

Microorganism	Inhibition zone[a] [mm]		
	UMY12	UMY14	UMY15
Aspergillus niger	2	2	2
Aspergillus flavus	1	2	3
Aspergillus terreus	1	2	2
Aspergillus fumigatus	1	3	3
Paecilomyces spp.	2	3	3
Mucor spp.	1	3	3
Trichoderma spp.	3	5	4
Bipolaris spp.	1	4	3

[a] Inhibition zones were measured from the edges of *M. pulcherrima* colonies to the beginning of the fungal lawns.

of the fungal species tested (Table II, Figs. 2B–D). However, germination and vegetative growth of *A. niger* retarded only for 3 days by pulcherrimin. After 3 days of incubation, *A. niger* overcame the antagonistic effect of pulcherrimin secreted by the *M. pulcherrima* strains. This may result from the growth features of *A. niger* hyphae. They might acquire soluble iron from outside of the pulcherrimin pigment zone and transport it to the other parts of the hyphae (Sipiczki, 2006). Alternatively, secretion and accumulation of an unknown substance from the germinating *A. niger* spores or hyphae might overcome the chelation of iron by the pigment pulcherrimin.

It is known that the treatment of microbial infections with antibiotics and fungicidal drugs results in the development of resistance (Espinel-Ingroff, 2008; Steffens *et al.*, 1996). The pigment pulcherrimin produced by different strains of *M. pulcherrima* can be a good alternative for topical applications in the prevention of certain bacterial, yeast and fungal infections in humans.

Acknowledgements

This work was supported by a research grant from The Scientific and Technological Research Council of Turkey (TUBİTAK, Project no. TOVAG 104O270).

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