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ORIGINAL PAPER

Poly(β-L-malic acid) production by diverse phylogenetic clades of *Aureobasidium pullulans*

Pennapa Manitchotpisit · Christopher D. Skory · Stephen W. Peterson · Neil P. J. Price · Karl E. Vermillion · Timothy D. Leathers

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Abstract Poly(β-L-malic acid) (PMA) is a natural biopolyester that has pharmaceutical applications and other potential uses. In this study, we examined PMA production by 56 strains of the fungus *Aureobasidium pullulans* representing genetically diverse phylogenetic clades. Thirty-six strains were isolated from various locations in Iceland and Thailand. All strains from Iceland belonged to a newly recognized clade 13, while strains from Thailand were distributed among 8 other clades, including a novel clade 14. Thirty of these isolates, along with 26 previously described strains, were examined for PMA production in medium containing 5% glucose. Most strains produced at least 4 g PMA/L, and several strains in clades 9, 11, and 13 made 9–11 g PMA/L. Strains also produced both pullulan and heavy oil, but PMA isolated by differential precipitation in ethanol exhibited up to 72% purity with no more than 12% contamination by pullulan. The molecular weight of PMA from *A. pullulans* ranged from 5.1 to 7.9 kDa. Results indicate that certain genetic groups of *A. pullulans* are promising for the production of PMA.

Keywords *Aureobasidium pullulans* · Heavy oil · Poly(β-L-malic acid) · Pullulan

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P. Manitchotpisit
Biochemistry Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Muangake, Lakhok, Patumthani 12000, Thailand

C. D. Skory · N. P. J. Price · T. D. Leathers (✉)
Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA
e-mail: tim.leathers@ars.usda.gov

S. W. Peterson
Bacterial Foodborne Pathogens and Mycology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA

K. E. Vermillion
Crop Bioprotection Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA

Introduction

Biopolymers have the potential to replace petroleum-derived materials in numerous applications as bioplastics, biocomposites, adhesives, films or fibers. First-generation bioplastics include the familiar biopolymers poly(β-lactic acid) (PLA) and poly(3-hydroxyalkanoates) (PHA). Although a variety of diverse microbial polyesters is known, research is needed to discover and develop promising second-generation biomaterials [12, 20]. One such biopolymer of interest is poly(β-malic acid) (PMA). Because it is biocompatible, degradable, and water soluble, PMA has primarily been studied for biomedical uses as a drug carrier [1, 11]. However, water-insoluble PMA derivatives have been used to make various solid devices, compression molded pellets, films, microparticles and nanoparticles, although to date none commercially [23].

PMA was first identified by Shimada et al. [18] as a protease inhibitor from *Penicillium cyclopium* and later from

the myxomycete (slime mold) *Physarum polycephalum* [3, 5], where it was determined to be a DNA polymerase inhibitor. *P. polycephalum* currently is used to produce PMA for pharmaceutical applications [11]. Nagata et al. [15] reported that isolates of the fungus *Aureobasidium* also produce PMA in high yields. In a small screening study, Liu and Steinbuchel [10] reported that 4 of 8 strains of *Aureobasidium* produced up to 1.1 g PMA/L prior to optimization. These authors suggested that if PMA could be produced more economically, it might find applications in the production of detergents, biodegradable plastics, or other biomaterials.

Aureobasidium pullulans is a polymorphic fungus, considered to be a filamentous ascomycete in class Dothideomycetes, subclass Dothideomycetidae [4, 17]. *A. pullulans* is well known as the source of the commercial polysaccharide, pullulan [8, 19]. The fungus also produces numerous other valuable bioproducts, including industrial enzymes, PMA, and a novel heavy oil [2, 7, 15]. We recently completed a multilocus molecular phylogeny of *A. pullulans* [13]. Interestingly, certain phylogenetically defined clades produced high levels of specific bioproducts, including pullulan, xylanase, and heavy oil [13, 14]. The objective of this study is to examine PMA production by genetically diverse phylogenetic clades of *A. pullulans,* in an effort to identity groups that produce high levels of PMA.

Materials and methods

A. pullulans isolates and classification

Thirty-six strains were isolated for this study between October 2009 and February 2010, designated RSU strains (Table 1). RSU strains 1–8 were collected from Iceland, with the remaining RSU strains collected from various provinces in Thailand. The method of isolation and phylogenetic analysis was described by Manitchotpisit et al. [13]. All isolates were classified using morphological characteristics and DNA sequence analyses of two loci, ITS and β-tubulin (*BT2*). All sequences were determined from bidirectional sequencing and aligned with our previous sequence data using ClustalW [22]. *Aureobasidium* sp. strain CU 30 was chosen as an outgroup species based on our previous studies [13]. Maximum parsimony analysis was performed using PAUP* [21] version 4.0b10. DNA sequences determined in this study were deposited in GenBank under accession numbers JF419588-JF419659, JF682345-JF682347, FJ150905.1, FJ150906.1, FJ157867.1, and FJ157869.1. Other strains used in this study were previously described [13] or obtained from the ARS Culture Collection, Peoria, IL (NRRL strains) or the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (CBS strains).

PMA production by strains of *A. pullulans*

A screen of PMA production by 56 strains of *A. pullulans* was conducted by culturing each isolate in 10 mL of modified pullulan production medium [13] containing 5% (w/v) glucose, 0.06% (w/v) peptone, 0.04% (w/v) yeast extract, 0.5% (w/v) K_2HPO_4, 0.04% (w/v) $MgSO_4.7H_2O$, and 0.1% (w/v) NaCl, pH 6.5, in 50 mL flasks cultured for 7 days at 25°C with shaking at 200 rpm. Cells were removed by centrifugation at 8,000 rpm (10,000 × g) for 20 min. Culture supernatants were analyzed for PMA and pullulan by analyzing malic acid and glucose yields, respectively, before and after acid hydrolysis. Samples (500 µL) of each supernatant were mixed with an equal volume of 1.0 M H_2SO_4 and hydrolyzed by incubation at 90°C for 9 h. Hydrolyzed samples and unhydrolyzed controls were diluted 1:5 in an HPLC dilution buffer (19 mM nitric acid and 34 mM propionic acid, which served as a microbiocide and internal standard, respectively) and applied to an Aminex HPX-87H column (Bio-Rad, Hercules, CA) equilibrated with 15 mM nitric acid at a flow rate of 0.5 mL/min at 60°C. Eluate from the column was analyzed by an RI detector. Increases in free malic acid and glucose in hydrolyzed samples were interpreted as representing PMA and pullulan, respectively. Pullulan (Showa Denko, Tokyo, Japan) and purified PMA (the kind gift of Dr. Eggehard Holler) from *P. polycephalum* (M_w 30,000) served as controls. The experiments were performed in triplicate and standard errors are reported.

Comparison of PMA, pullulan, and heavy oil production

To study potential relationships among PMA, pullulan and heavy oil production, representative strains were grown for 7 days in 50 mL of modified pullulan production medium in 250 mL flasks. The pH and OD_{600} of each culture were recorded, and cultures were centrifuged at 8,000 rpm (10,000 × g) for 20 min to prepare supernatants. Samples (1 mL) were retained for PMA and pullulan determinations using HPLC as described above. The remaining culture supernatants were adjusted to pH 5.0 by the dropwise addition of 30% (w/v) $CaCO_3$. Pullulan was precipitated by the addition of one volume of 95% ethanol followed by centrifugation at 10,000 rpm (16,900 × g) for 30 min. PMA was then precipitated by the addition of an additional volume of 95% ethanol, followed by incubation at 4°C overnight and centrifugation at 10,000 rpm (16,900 × g) for 30 min. Precipitated PMA was dissolved in 200 mL of Milli-Q water (Millipore, Billerica, MA) and lyophilized. Heavy oil was extracted from the cell pellets using methyl ethyl ketone as

Table 1 New strains of *Aureobasidium pullulans* isolated in this study

Isolate	NRRL number	Clade	Host/source	Collection site and date
RSU 1	NRRL 62024	13	Leaf	Vik, Iceland (October, 2009)
RSU 2	NRRL 62025	13	Leaf	Vik, Iceland (October, 2009)
RSU 3	NRRL 62026	13	Leaf	Vik, Iceland (October, 2009)
RSU 4	NRRL 62027	13	Leaf	Geysir, Haukadalur Valley, Iceland (October, 2009)
RSU 5	NRRL 62028	13	Leaf	Þingvellir, Iceland (October, 2009)
RSU 6	NRRL 50383	13	Leaf	Reykjavik, Iceland (October, 2010)
RSU 7	NRRL 62029	13	Leaf	Reykjavik, Iceland (October, 2009)
RSU 8	NRRL 62030	13	Leaf	Reykjavik, Iceland (November, 2009)
RSU 9	NRRL 62031	11	Leaf	Nakornratchasima, Thailand (January, 2010)
RSU 10	NRRL 62032	1 or 2	Leaf	Chiangmai, Thailand (January, 2010)
RSU 11	NRRL 62033	14	Leaf	Chiangmai, Thailand (January, 2010)
RSU 12	NRRL 50381	5	Leaf	Chiyaphum, Thailand (January, 2010)
RSU 13	NRRL 62034	9	Leaf	Chonburi, Thailand (February, 2010)
RSU 14	NRRL 62035	3	Leaf	Chonburi, Thailand (February, 2010)
RSU 15	NRRL 62036	14	Leaf	Ratchaburi, Thailand (February, 2010)
RSU 16	NRRL 62037	7	Leaf	Karnchanaburi, Thailand (February, 2010)
RSU 17	NRRL 62038	9	Leaf	Patalung, Thailand (February, 2010)
RSU 18	NRRL 62039	9	Leaf	Chonburi, Thailand (February, 2010)
RSU 19	NRRL 62040	9	Leaf	Patalung, Thailand (February, 2010)
RSU 20	NRRL 62041	8	Leaf	Patalung, Thailand (February, 2010)
RSU 21	NRRL 62042	11	Leaf	Patalung, Thailand (February, 2010)
RSU 22	NRRL 62043	3	Leaf	Patumthani, Thailand (January, 2010)
RSU 23	NRRL 62044	3	Leaf	Patumthani, Thailand (January, 2010)
RSU 24	NRRL 62045	3	Leaf	Patumthani, Thailand (January, 2010)
RSU 25	NRRL 62046	7	Painted wall	Patumthani, Thailand (January, 2010)
RSU 26	NRRL 62047	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 27	NRRL 62048	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 28	NRRL 62049	7	Grout	Patumthani, Thailand (February, 2010)
RSU 29	NRRL 50384	11	Leaf	Bangkok, Thailand (February, 2010)
RSU 30	NRRL 62050	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 31	NRRL 62051	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 32	NRRL 50382	8	Leaf	Bangkok, Thailand (February, 2010)
RSU 33	NRRL 62052	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 34	NRRL 62053	3	Leaf	Bangkok, Thailand (February, 2010)
RSU 35	NRRL 62054	1 or 2	Leaf	Bangkok, Thailand (February, 2010)
RSU 36	NRRL 62055	3	Leaf	Bangkok, Thailand (February, 2010)

described by Manitchotpisit et al. [14]. The experiments were performed in triplicate and standard errors are reported.

Characterization of isolated PMA

Lyophilized PMA samples were dissolved in Milli-Q water at 1.0% (w/v) and assayed for PMA content by HPLC as described above. For analysis by nuclear magnetic resonance spectroscopy (NMR), samples were dissolved in D_2O to a final concentration of 2 mg/mL and analyzed by ^{1}H-NMR using a Bruker Avance 500 spectrometer (Bruker Biospin Corp., Billerica, MA) equipped with a 5 mm broadband inverse (BBI) probe with z-gradient. NMR data were processed with Topspin v1.3 software and the chemical shifts were reported as parts per million from external tetramethylsilane (TMS) based on the lock signal. Pullulan (Showa Denko) and purified PMA from *P. polycephalum* (M_w 30,000) served as NMR standards. Molecular weight distributions were analyzed by size exclusion chromatography, using a Shodex SB-806 M HQ column (Showa Denko,

Tokyo, Japan) eluted with 0.05 M sodium nitrate at room temperature and a flow rate of 0.5 mL/min. Separations were monitored using a Shodex OR-1 optical rotation detector (Showa Denko K.K.). Pullulan standards of varying known sizes (Showa Denko) were used to estimate the molecular weight of PMA.

Results and discussion

Isolation and classification of *A. pullulans* strains

Thirty-six strains of *A. pullulans* were isolated for this study, designated as RSU strains (Table 1). RSU strains 1–8 were collected in Iceland, and RSU strains 9–36 were collected from various provinces in Thailand, between October, 2009 and February, 2010. Most strains were isolated from leaf samples, consistent with the reputation of *A. pullulans* as a common epiphyte. All new isolates were deposited in the ARS Culture Collection, National Center for Agricultural Utilization Research, USDA, Peoria, IL, USA (accession numbers are shown in Table 1). New strains were classified using morphological characteristics and by DNA sequence analyses of the ITS and *BT2* loci.

Phylogenetic analysis of the ITS region is useful to distinguish *A. pullulans* from other species [13], and by this measure all 36 new strains were confirmed as *A. pullulans*. However, ITS analysis cannot be used to differentiate

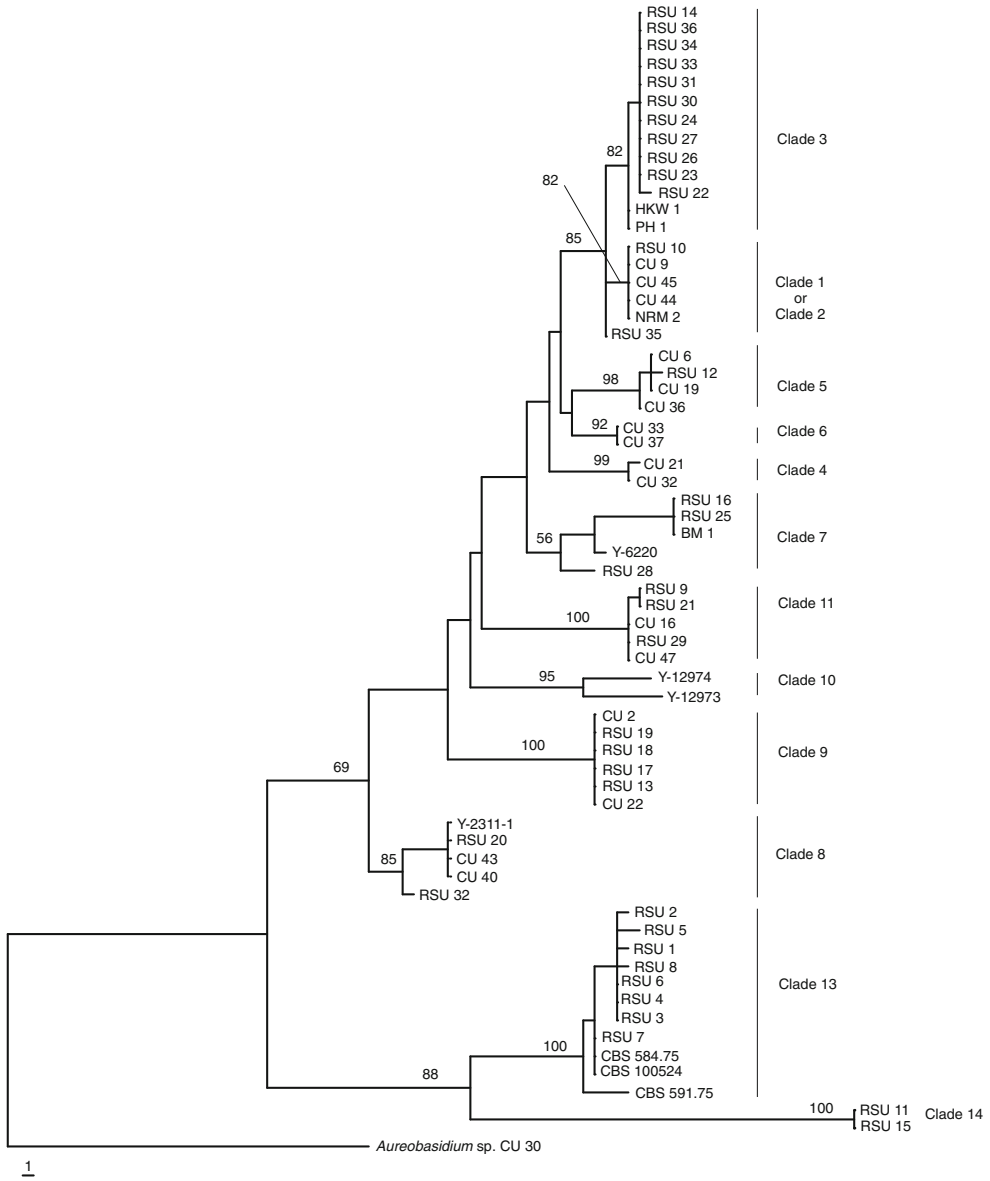

Fig. 1 Maximum parsimony tree of *Aureobasidium pullulans* isolates calculated with PAUP*. The bootstrap proportion from 1,000 repetitions appears above nodes. The clade numbering system is from the multilocus phylogenetic study of Manitchotpisit et al. [13]

individual isolates. On the other hand, *BT2* sequences are useful to classify *A. pullulans* isolates into phylogenetic groups [13]. The *BT2* region is convenient for this analysis because it involves relatively short PCR products (approximately 450 bp) and only one pair of primers is required [13]. Most of these isolates were classified into distinct phylogenetic clades, although strains RSU 10 and RSU 35 were assigned to clade 1 or 2. Fig. 1 shows a maximum parsimony tree of *BT2* sequences of 63 *A. pullulans* strains, including the new isolates. The clade numbering system shown is based on our previous multilocus phylogenetic study [13]. Eight strains isolated from Iceland (RSU 1–8) all belong to newly recognized clade 13, which contains only strains from temperate climates. Clade 13 includes CBS strain 584.75, which is the exneotype strain for *A. pullulans var. pullulans*, as well as CBS strain 100,524, now considered *A. pullulans var. pullulans* [24]. Also included in clade 13 is CBS strain 591.75, identified as the best PMA producer among 8 strains examined by Liu and Steinbuchel [10]. Two new isolates from Thailand, strains RSU 11 and RSU 15, belong to a novel clade 14 (Table 1). The remaining 26 new isolates from Thailand were distributed among 8 previously described phylogenetic clades (clades 1 and 2, 2 isolates; clade 3, 11 isolates; clade 5, 1 isolate; clade 7, 3 isolates; clade 8, 2 isolates; clade 9, 4 isolates; and clade 11, 3 isolates). The variety of these strains supports our previous conclusion that Thailand is a source of genetically diverse isolates of *A. pullulans* [13].

Phenotypic observations of the new isolates were consistent with previous descriptions of *A. pullulans* [13]. Colonial morphologies grown on yeast malt agar for 7 days generally were smooth, moist, yeast-like, and pale pink. Strain RSU 12 exhibited a dark vinaceous pigment characteristic of clade 5. Isolates in clade 8 (RSU 20 and RSU 32) exhibited characteristic color rings of pale pink and orange. On malt extract agar plates, colonies generally consisted of white hyphae with dark olivaceous centers, except for isolates in clades 5, 8, and 11, which showed vinaceous, yellow, and white pigments, respectively. Microscopically, early cultures showed typical polymorphic forms of *A. pullulans* with blastospores, swollen cells, and pseudohyphae, with later cultures showing hyphae and chlamydospores.

PMA production by diverse phylogenetic clades of *A. pullulans*

Fifty-six strains of *A. pullulans* were examined for PMA production, including 30 of the new isolates described above and an additional 26 strains representing diverse clades of *A. pullulans* (Table 2). In preliminary studies, we tested the mineral salts medium used by Nagata et al. [15] and Liu and Steinbuchel [10]. However, we obtained higher

Table 2 Poly(β-L-malic acid) (PMA) production by *Aureobasidium pullulans*

Clade	Strain	NRRL number	PMA (g/L)
1	CU 44	NRRL 58555	4.2 ± 0.3
	CU 45	NRRL 58556	4.1 ± 0.1
2	CU 9	NRRL 58522	4.7 ± 0.2
	NRM2	NRRL 58560	4.5 ± 0.0
1 or 2	RSU 10	NRRL 62032	8.9 ± 0.3
	RSU 35	NRRL 62054	8.8 ± 0.1
3	PH1	NRRL 58563	4.1 ± 0.1
	HKW1	NRRL 58562	4.2 ± 0.1
	RSU 14	NRRL 62035	8.1 ± 0.2
	RSU 22	NRRL 62043	8.6 ± 0.5
	RSU 23	NRRL 62044	7.6 ± 0.3
	RSU 26	NRRL 62047	7.6 ± 0.2
	RSU 27	NRRL 62048	7.2 ± 0.1
4	CU 21	NRRL 58534	6.8 ± 0.2
	CU 32	NRRL 58545	6.1 ± 0.3
5	CU 6	NRRL 58519	4.5 ± 0.1
	CU 36	NRRL 58548	5.0 ± 0.5
	RSU 12	NRRL 50381	7.9 ± 0.5
6	CU 33	NRRL 58546	5.0 ± 0.1
	CU 37	NRRL 58549	7.0 ± 0.2
7	Y-6220	NRRL Y-6220	3.9 ± 0.0
	BM1	NRRL 58561	4.7 ± 0.1
	RSU 16	NRRL 62037	6.3 ± 0.8
	RSU 25	NRRL 62046	5.7 ± 0.2
	RSU 28	NRRL 62049	10 ± 1.6
8	Y-2311-1	NRRL Y-2311-1	6.2 ± 0.3
	CU 40	NRRL 58552	6.0 ± 0.2
	CU 43	NRRL 50380	6.8 ± 0.3
	RSU 20	NRRL 62041	8.8 ± 0.2
	RSU 32	NRRL 50382	9.7 ± 0.5
9	CU 2	NRRL 58515	9.3 ± 0.6
	CU 22	NRRL 58535	4.4 ± 0.2
	RSU 13	NRRL 62034	8.6 ± 0.1
	RSU 17	NRRL 62038	9.6 ± 0.4
	RSU 18	NRRL 62039	8.1 ± 0.3
	RSU 19	NRRL 62040	8.6 ± 0.1
10	Y-12973	NRRL Y-12973	2.6 ± 0.2
	Y-12974	NRRL Y-12974	6.5 ± 0.3
11	CU 16	NRRL 58529	6.5 ± 0.3
	CU 47	NRRL 58558	5.2 ± 0.1
	RSU 9	NRRL 62031	9.9 ± 0.1
	RSU 21	NRRL 62042	8.7 ± 0.2
	RSU 29	NRRL 50384	11 ± 0.9
13	CBS 584.75	NRRL 58012	7.3 ± 0.2
	CBS 100524	NRRL 58013	7.8 ± 0.3
	RSU 1	NRRL 62024	8.4 ± 0.1
	RSU 2	NRRL 62025	8.0 ± 0.5

Table 2 continued

Clade	Strain	NRRL number	PMA (g/L)
	RSU 3	NRRL 62026	7.1 ± 0.1
	RSU 4	NRRL 62027	8.1 ± 0.4
	RSU 5	NRRL 62028	8.6 ± 0.3
	RSU 6	NRRL 50383	11 ± 0.0
	RSU 7	NRRL 62029	10 ± 0.5
	RSU 8	NRRL 62030	7.3 ± 0.3
	CBS 591.75	NRRL 62056	8.8 ± 0.4
14	RSU 11	NRRL 62033	0.0 ± 0.0
	RSU 15	NRRL 62036	3.6 ± 0.1

Grown for 7 days in 10 mL of modified pullulan production medium

yields of PMA from a modified pullulan production medium, subsequently used in these studies. Most strains produced PMA on this medium, and several strains in clades 9, 11, and 13 made 9–11 g PMA/L (Table 2). Clade 13 strain CBS 591.75, previously described as the best producer among eight strains of *A. pullulans* [10], produced 8.8 g PMA/L under these conditions.

Comparison of PMA, pullulan, and heavy oil production

PMA, pullulan, and heavy oil production were compared from strains associated with nine different clades (Table 3). Cultures were grown on a slightly larger scale (50 mL) to provide greater amounts of material for analysis. Growth yields varied considerably and were not obviously related to PMA yields. PMA yields also were somewhat different from those observed in smaller cultures, presumably due to the effects of scale. Nevertheless, strain RSU 7 from clade 13 was the best producer of PMA, consistent with results from the screening study. Only strain RSU 15 failed to produce PMA under these conditions, consistent with observations of low production by members of clade 14.

All strains produced both pullulan and heavy oil (Table 3). Strain NRRL Y-12974 was previously described as a high-level producer of pullulan [13], and new isolates RSU 7 and RSU 15 produced nearly 10 g pullulan/L. Strains CU 44 and CU 47 produced somewhat less pullulan than previously reported [13], possibly because of differences in growth and assay conditions. Strain CU 47 (clade 11) gave the highest yields of heavy oil. In most cases, the color of the oil produced corresponded to the color of the culture (Table 3). No relationships were apparent among the yields of PMA, pullulan, and oil. However, in preliminary experiments we observed that the addition of 3.0% (w/v) $CaCO_3$ to growing cultures increased yields of PMA by 25–80% and decreased yields of both pullulan and oil (data not shown). Liu and Steinbuchel [10] cultured *A. pullulans* in medium without $CaCO_3$, but added $CaCO_3$ to culture supernatants to facilitate precipitation of Ca-PMA. On the other hand, Nagata et al. [15] included $CaCO_3$ in the culture medium as a neutralizing agent, and Kurosawa et al. [6] later observed that the absence of $CaCO_3$ favored the accumulation of heavy oil instead of PMA. Although culture pH is stabilized by the addition of $CaCO_3$, it is not clear that pH is the sole factor influencing PMA production. Lee et al. [9] concluded that, in the slime mold *P. polycephalum*, $CaCO_3$ can serve as a significant carbon source for PMA biosynthesis.

Characterization of isolated PMA

PMA was isolated by differential precipitation in ethanol, which preferentially separates PMA from pullulan. The PMA and pullulan content was assayed as described above (Table 4). Strains CU 22, CU 44, and CU 47 exhibited PMA of about 70% purity, with no more than 8% pullulan. Despite the fact that strain NRRL Y-12974 produced a great deal of pullulan, precipitated PMA from this strain showed 62% purity with 12% pullulan. This suggests that differential precipitation is relatively successful at separating pullulan from PMA.

Table 3 Comparison of poly(*β*-L-malic acid) (PMA), pullulan, and oil production by *Aureobasidium pullulans*

Clade	Strain	pH	OD_{600}	PMA (g/L)	Pullulan (g/L)	Oil (g/L)	Culture color	Oil color
1	CU 44	3.7 ± 0.0	4.5 ± 0.3	7.7 ± 0.1	2.7 ± 0.5	1.5 ± 0.2	Light olivaceous	Yellow
3	RSU 26	4.1 ± 0.0	4.8 ± 0.2	5.7 ± 0.4	4.4 ± 0.6	0.3 ± 0.2	Dark olivaceous	Olivaceous
7	RSU 25	5.5 ± 0.2	3.5 ± 0.3	4.8 ± 0.3	1.8 ± 0.5	0.1 ± 0.1	Cream	Yellow
8	CU 43	3.7 ± 0.0	1.4 ± 0.2	7.1 ± 0.1	1.7 ± 0.4	1.3 ± 0.1	Cream	Orange-red
9	CU 22	3.8 ± 0.0	7.0 ± 0.3	6.8 ± 0.1	1.5 ± 0.2	1.1 ± 0.1	Orange	Dark olivaceous
10	Y-12974	4.0 ± 0.0	2.9 ± 0.2	6.7 ± 0.3	19 ± 1.1	1.9 ± 0.1	Light olivaceous	Yellow
11	CU 47	3.7 ± 0.0	5.5 ± 0.2	7.7 ± 0.1	4.6 ± 0.8	3.9 ± 0.2	Light olivaceous	Olivaceous
13	RSU 7	3.0 ± 0.0	3.7 ± 0.3	8.9 ± 0.1	9.8 ± 0.1	0.5 ± 0.1	Light olivaceous	Yellow
14	RSU 15	3.4 ± 0.0	3.9 ± 0.2	0.0 ± 0.0	9.5 ± 0.4	1.1 ± 0.1	Black	Olivaceous

Grown for 7 days in 50 mL of modified pullulan production medium

Table 4 Characterization of isolated PMA from *Aureobasidium pullulans*

Strain	%PMA	%Pullulan	PMA molecular weight (kDa)
CU 22	72	8	7.9
CU 43	57	2	7.2
CU 44	68	8	7.1
CU 47	70	7	7.7
NRRL Y-12974	62	12	6.4
RSU 7	57	6	5.1
Pullulan standard[a]	0	85	Not applicable
PMA standard[b]	96	4	27

Isolated by differential ethanol precipitation from supernatants of cultures grown for 7 days in 50 mL of modified pullulan production medium

[a] Showa Denko

[b] Poly(β-L-malic acid) from *Physarum polycephalum* (M_w 30,000)

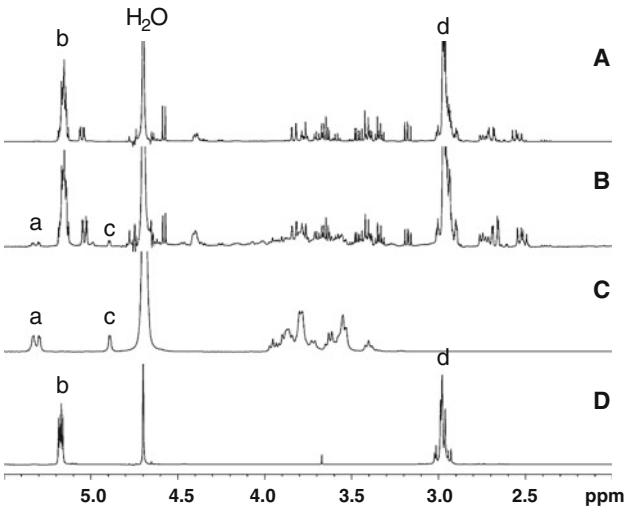

Fig. 2 ^{1}H-NMR spectra of isolated poly(malic acid) PMA from *Aureobasidium pullulans*. *A* Strain CU 22. *B* Strain NRRL Y-12974. *C* Pullulan standard. *D* PMA standard

Isolated PMA also was characterized by ^{1}H-NMR spectroscopy. Fig. 2 shows spectra obtained for strains NRRL Y-12974 and CU 22, along with purified pullulan and PMA standards. PMA is characterized by multiplets due to methylene (-CH_2-, peak b) and methine (-CH-, peak d) protons (panel D). Pullulan is characterized by peaks for the branched and unbranched alpha-1,4-linked (peak a) and the alpha-1,6-linked (peak c) glucosyl residues (panel C). Other pullulan signals at 3–4 ppm are due to the carbohydrate ring protons. Panel B of Fig. 2 shows that the preparation from strain NRRL Y-12974 contains PMA and likely some contaminating pullulan. Panel A shows the presence of PMA in the preparation from strain CU 22, and no contaminating pullulan is evident. However, the preparation may contain non-pullulan carbohydrate, as shown by anomeric proton peaks at 4.6 ppm and 5.1 ppm, and ring protons at 3.0-4.0 ppm. NMR spectra of strains CU 43, CU 44, CU 47, and RSU 7 appeared identical to that of CU 22 (data not shown).

We also examined the molecular weight of PMA produced by strains of *A. pullulans*. Nakajima-Kambe et al. [16] reported that the molecular weight of PMA from strain A-91 of *Aureobasidium* was 6-11 kDa. Similarly, Liu and Steinbuchel [10] reported a molecular weight of 3–5 kDa for PMA from *A. pullulans*, while PMA from the slime mold *P. polycephalum* is on the order of 50 kDa [11]. Consistent with these reports, we found that PMA from *A. pullulans* strains ranged from 5.1 to 7.9 kDa, while a PMA standard from *P. polycephalum* was 27 kDa (Table 4). Thus, isolated PMA preparations characterized in this study were all of relatively low molecular weight. It is possible that examination of additional strains will provide exceptions to this rule. In the case of the polysaccharide pullulan, it is clear that molecular weight varies greatly on a strain-specific basis [8].

In conclusion, most strains of *A. pullulans* in genetically diverse phylogenetic clades produced PMA, and certain clades included strains that produced relatively high levels of PMA (9–11 g/L). PMA isolated by differential precipitation in ethanol exhibited up to 72% purity with no more than 12% contamination by pullulan. The molecular weight of PMA from *A. pullulans* ranged from 5.1 to 7.9 kDa. Thus, certain genetic groups of *A. pullulans* are promising for the production of PMA.

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References

1. Braud C, Bunel C, Vert M (1985) Poly(β-malic acid): a new polymeric drug-carrier. Evidence for degradation in vitro. Polym Bull 13:293–299
2. Deshpande MS, Rale VB, Lynch JM (1992) *Aureobasidium pullulans* in applied microbiology: a status report. Enzyme Microbial Technol 14:514–527
3. Fischer H, Erdmann S, Holler E (1989) An unusual polyanion from *Physarum polycephalum* that inhibits homologous DNA polymerase in vitro. Biochem 28:5219–5226
4. Hibbett DS et al (2007) A higher-level phylogenetic classification of the fungi. Mycol Res 111:509–547
5. Holler E, Angerer B, Achhammer G, Miller S, Windisch C (1992) Biological and biosynthetic properties of poly-L-malate. FEMS Microbiol Rev 103:109–118
6. Kurosawa T, Sakai K, Nakahara T, Oshima Y, Tabuchi T (1994) Extracellular accumulation of the polyol lipids, 3, 5-dihydroxydecanoyl and 5-hydroxy-2-decenoyl esters of arabitol and mannitol, by Aureobasidium sp. Biosci Biotech Biochem 58:2057–2060

7. Leathers TD (1989) Purification and properties of xylanase from *Aureobasidium*. J Ind Microbiol 4:341–348
8. Leathers TD (2002) Pullulan. In: Vandamme EJ, De Baets S, Steinbuchel A (eds) Biopolymers, vol 6, polysaccharides II: polysaccharides from eukaryotes. Wiley-VCH, Weinheim, pp 1–35
9. Lee BS, Maurer T, Kalbitzer HR, Holler E (1999) β-Poly(L-malate) production by *Physarum polycephalum*: ^{13}C Nuclear magnetic resonance studies. Appl Microbiol Biotechnol 52:415–420
10. Liu S, Steinbuchel A (1996) Investigation of poly(β-L-malic acid) production by strains of *Aureobasidium pullulans*. Appl Microbiol Biotechnol 46:273–278
11. Ljubimova JY, Fujita M, Khazenzon NM, Lee BS, Wachsmann-Hogiu S, Farkas DL, Black KL, Holler E (2008) Nanoconjugate based on polymalic acid for tumor targeting. Chemico-Biol Interact 171:195–203
12. Luengo JM, Garcia B, Sandoval A, Naharro G, Olivera ER (2003) Bioplastics from microorganisms. Curr Opin Microbiol 6:251–260
13. Manitchotpisit P, Leathers TD, Peterson SW, Kurtzman CP, Li X-L, Eveleigh DE, Lotrakul P, Prasongsuk S, Dunlap CA, Vermillion KE, Punnapayak H (2009) Multilocus phylogenetic analyses, pullulan production and xylanase activity of tropical isolates of *Aureobasidium pullulans*. Mycol Res 113:1107–1120
14. Manitchotpisit P, Price NPJ, Leathers TD, Punnapayak H (2011) Heavy oils produced by *Aureobasidium pullulans*. Biotechnol Lett 33:1151–1157
15. Nagata N, Nakahara T, Tahuchi T (1993) Fermentative production of poly(β-L-malic acid), a polyelectronic biopolyester by *Aureobasidium* sp. Biosci Biotech Biochem 57:638–642
16. Nakajima-Kambe T, Hirotani N, Nakahara T (1996) Poly(beta-malic acid) production by the non-growing cells of *Aureobasidium* sp strain A-91. J Ferm Bioeng 82:411–413
17. Schoch CL, Shoemaker RA, Seifert KA, Hambleton S, Spatafora JW, Crous PW (2006) A multigene phylogeny of the *Dothideomycetes* using four nuclear loci. Mycologia 98:1041–1052
18. Shimada K, Matsushi K, Fukumoto J, Yamamoto T (1969) Poly-(L)-malic acid—a new protease inhibitor from *Penicillium cyclopium*. Biochem Biophy Res Commun 35:619–624
19. Singh RS, Saini GK, Kennedy JF (2008) Pullulan: microbial sources, production and applications. Carbohydr Polym 73:515–531
20. Steinbuchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228
21. Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony, version 4.0b10. Sinauer Associates, Sunderland
22. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
23. Vert M (1998) Chemical routes to poly(β-malic acid) and potential applications of this water-soluble bioresorbable poly(β-hydroxy alkanoate). Polym Degrad Stab 59:169–175
24. Zalar P, Gostincar C, de Hoog GS, Ursic V, Sudhadham M, Gunde-Cimerman N (2008) Redefinition of *Aureobasidium pullulans* and its varieties. Stud Mycol 61:21–38