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ORIGINAL PAPER

Biodiversity of Bacteria Associated with Eight *Pleurotus ostreatus* (Fr.) P. Kumm. Strains from Poland, Japan and the USA

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Abstract

Few publications report the occurrence of bacteria associated with fungal cells. The presence of bacteria associated with one strain of *Pleurotus ostreatus* (Fr.) P. Kumm. was described in the literature. We describe the biodiversity of bacteria associated with eight oyster mushroom strains from Japan, Poland, and the USA. The presence of microorganisms associated with all tested *P. ostreatus* strains was confirmed using fluorescent microscopy. Among 307 sequences, 233 of clones representing 34 genera and 74 sequences were identified as *Bacteria*. Most of the bacteria associated with the strain PUSAS were related to *E. coli* and two clones were related to *Cupriavidus* genus. The biodiversity of clones isolated from fungal strains originating from Japan and Poland ranged from 15 to 32 different bacterial clones. The most often the bacteria related to genus *Curvibacter*, *Pseudomonas*, *Bacillus*, *Cupriavidus*, *Pelomonas*, and *Propionibacterium* were associated with the strains of fungi mentioned above. Laccase-like (LMCO) genes were identified in whole bacterial DNA isolated from the associated bacteria but β-glucosidase and β-xylanase genes were not detected.

Key words: *Pleurotus ostreatus*, biodiversity, associated bacteria, fluorescent microscopy, 16S rRNA PCR

Introduction

Various types of relationship between two or more organisms are well known among plants and in the animal kingdom. However, little is known about the relationship and interactions between fungi and bacteria. There are three most common types of these relationships. Bacteria can live in the same environment as fungi where both organisms live close to each other but not in direct contact. This kind of relationship is common in various environments like forests, fermented food or animal tissues affected by diseases (Frey-Klett et al. 2011). More complicated type of bacterial-fungal association is mixed biofilms, where bacteria may live on fungal hyphae and are held by the extracellular matrix formed from molecules secreted by both organisms (Donlan and Costerton 2002). This kind of coexistence can be found in medical equipment (Pierce 2005) as well as in some infections (Hogan et al. 2007) or mycorrhizal systems (Sarand et al. 1998). The third type of interactions between these two groups of organisms is endosymbiosis when bacteria live inside fungal cells and do not produce any specific structures. It is the most complex type of relationship and often, in contrast to free-living fungi-associated bacteria it is impossible to cultivate such bacteria outside a fungal host. The presence of unculturable endosymbiotic bacteria was described mostly in cells of arbuscular mycorrhizal fungi belonging to *Gigasporaceae* family (Bianciotto et al. 1996, 2000, 2003; Bonfante 2003; Cruz et al. 2008). The endosymbiotic bacteria were also observed in the cells of pathogenic fungus *Rhizopus microsporus* although in this case, authors were able to grow isolated bacteria on microbiological media (Partida-Martinez et al. 2007a; 2007b). Among *Basidiomycota* such relationship was found in *Laccaria bicolor*. Similarly to *Gigaporaceae* endosymbionts, these bacteria were also unculturable (Bertaux et al. 2003). Only one report described bacteria of *Burkholderia cepacia* complex related with oyster mushroom *P. ostreatus*. Authors, however, did not establish if these bacteria are endosymbionts or just fungi-related organisms (Yara et al. 2006).

The aim of this work was to identify bacteria associated with eight *P. ostreatus* strains, and also the

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description of their biodiversity and assessment of their ability for N_2 fixation, cellulose, xylanase, and laccase-like multicopper oxidase activity.

Experimental

Materials and Methods

Fungal strains. Eight strains from different geographical zones (PB63S, PBo6S, PxS, P234, P112 – Poland; PB7'96, PB8 – Japan, PUSAS – the USA) of *P. ostreatus* were analyzed. All strains were shared by the Department of Fruit, Vegetable and Mushroom Technology at the University of Life Sciences in Lublin. According to our knowledge, the strains analyzed were never cultivated on media containing antibiotics. Before experiments the fungal strains were cultivated for seven days in 28°C on Petri dishes with onion medium (onion extract 1000 ml (150 g of chopped onion boiled in 500 ml distilled H_2O, filtered on cotton filter and filled with distilled H_2O to 1000 ml, glucose 20 g, peptone 3 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, agarose 20 g).

Observation of bacteria-like organisms associated with *P. ostreatus*. Observation of living bacteria-like organisms associated with fungal cells was carried out using a fluorescent microscope and a set of fluorescent dyes (Bianciotto et al. 2000). Total fungal mycelium from one Petri dish of every oyster mushroom strain cultivated for seven days was collected and grounded in a sterile glass mortar in 1 ml of sterile 0.1 M $MgSO_4$. The obtained suspension was stained in a 50-µl volume with the fluorescent dye (Viability/Cytotoxicity Assay Kit for Bacteria, Biotium, USA) according to the manufacturer's protocol. Observations were made using Nikon Eclipse 90i fluorescent microscope with the Omega XF25 filter. Pictures and videos were taken in the same conditions. The fluorescent kit used in the experiments is composed of two dyes. DMAO migrates through a cell membrane and stains double-stranded DNA of both living and dead bacteria on green or yellow-green color. The second dye, EthD-III migrates through damaged membranes of dead cells only and stains bacterial DNA on red color. This dye is stronger than DMAO and covers its color, thus dead bacteria are visible as red and live bacteria are visible as green or yellow-green organisms.

Isolation of organisms associated with *P. ostreatus*. Isolation of organisms associated with fungal strains was done from seven-days old fungal culture. The 16 mm diameter roundel was picked and placed mycelium towards to the medium on base and modified solid TSB medium (Sigma-Aldrich, USA), called later in this article as TSAt. The modification consisted of the addition of 1% of Tween 20 to the medium (ApliChem GmbH, Germany) as described by Yara et al. (2006). After 14 days of cultivation in 28°C, 25-mm^2 square area of the solid medium with visible microcolonies was picked and placed in 50 ml of liquid TSBt medium (TSB with 1% of Tween 20) in Erlenmeyer flasks. Samples were cultivated for 14 days in 28°C stationary. After cultivation TSAt fragment was removed from samples and samples were centrifuged in 10 000 × g for 10 minutes. The supernatant was poured out and the precipitate was used for further experiments.

DNA isolation. Genomic DNA was extracted from precipitates using Genomic Mini AX Bacteria (A&A Biotechnology, Poland) according to the instructions of the manufacturer. The obtained DNA was purified with PowerClean DNA Clean-Up Kit (MO BIO Laboratories, Inc., USA) in accordance with the manufacturer's protocol. Purified DNA was stored at –20°C for further experiments.

The identification of the associated organisms was performed using whole DNA isolated from fungal strains. Fresh fungal mycelia were harvested from 25 mm^2 square area of the solid medium of 7 days old fungal colonies, then suspended and rubbed in 1 ml of 0.1 M $MgSO_4$ in sterile glass mortars. Suspension in a volume of 100 µl of each fungal strain was used for DNA isolation using PowerSoil DNA Isolation Kit (MO-BIO Laboraories, Inc., USA) according to the manufacturer's protocol. DNA was purified as described above.

16S rRNA amplification and identification. The confirmation of the bacterial nature of isolated microcolonies was carried out by determining the size of the 16S rRNA gene fragment by PCR. The reaction mixture contained isolated DNA, 2 × PCR RED Master Mix (DNA-GDANSK, Poland) kit, 799f, and 1492r primers, according to a procedure designed to distinguish bacteria and plant mitochondrial the 16S rRNA gene fragment as described by Chelius and Triplett (2001). DNA from *Pseudomonas fluorescens* PE16 strain was used as bacterial control. DNA isolated from maize cv. Cyrkon (*Zea mays* L.) was used as plant control. The controls were shared by the Agriculture Microbiology Lab at the Wrocław University of Environmental and Life Sciences. The electrophoresis of the products obtained was performed (70 min, 100 V) on the agarose gel (0.8%) with 5% ethidium bromide. Bands were visualized in Dark Hood DH 40/50 and analyzed with Gelix-One 1D Software (Biostep GmbH, Germany).

The identification of bacteria was performed using a molecular cloning technique. Products of 16S rRNA PCR were purified with PCR SureClean Plus (Bioline LTD., UK) kit. Cloning was carried out with Zero Blunt PCR Cloning Kit (Life Technologies, USA) according to manufacturer's protocol. The ligation mixture was prepared in 50:1 insert:vector ratio (250 ng of purified product), the ligation step was 30 minutes long. Competent *E. coli* TOP10 cells (Life Technologies,

Table I
Accessing numbers of *P. ostreatus* clones sequences deposited in NCBI database with most similar strains from NCBI database.

NCBI accessing number	Parent strain	Number of clones	The most similar strains in NCBI database	
			Strain name	Accessing number and similarity (%)
KR779716	PxS; PBo6S	2	*Methylobacterium* sp. 63	JF905617 (99)
KR779718	PBo6S	1	*Corynebacterium ureicelerivorans* IMMIB RIV-2301	CP009215 (99)
KR779719	PBo6S	1	*Corynebacterium* sp. clone YHSS1	EF658675 (100)
KR779720	PB8	1	*Corynebacterium* sp. clone OD-12	KX379256 (100)
KR779721	PxS; PBo6S; PB7'96	4	*Weissella cibaria* BM2	CP027427 (99)
MH424523	P112	2	*Weissella confusa* SM10	KU060300 (100)
KR779722	PBo6S; PB63S; P234; PxS	5	*Paracoccus* sp. clone SL36	HQ264096 (100)
MH424529	PB7'96	1	*Paracoccus yeei* TWCC 57946	LC371258 (100)
KR779723	P234	1	*Bacillus* sp. 1NLA3E	CP005586 (99)
KR779725	PB7'96	1	*Bacillus* sp. R-66632	KT185191 (99)
KR779726	P234; PB8; P112; PBo6S	8	*Bacillus* sp. CHORDb1	MG995009 (100)
MH424530	P112	2	*Bacillus* sp. clone D	JX505089 (99)
MH424531	PB8	1	*Bacillus megaterium* Y103	MH368091 (99)
KR779727	PxS	1	*Nocardioides sp*. clone EHFS1_S02a	EU071473 (100)
KR779728	PBo6S; PB7'96	4	*Nocardioides terrigena* DS-17	NR_044185 (99)
KR779729	PxS	1	*Citricoccus* sp. PL13f_S6	JF274870 (99)
KR779730	PxS	1	*Micrococcus* sp. EF1B-B144	KC545358 (99)
MH424533	P234	1	*Micrococcus luteus* JGTA-S5	KT805418 (99)
MH424534	PB8	1	*Micrococcus* sp. cpRA422	KJ510213 (100)
MH424535	P112	1	*Micrococcus* sp. strain CAU1456	MG214549 (99)
MH424536	P234; PxS	2	*Micrococcus terreus* IHBB 9339	KU921566 (99)
KR779731	PxS; P112; PB7'96; P234; PB8	9	*Pelomonas saccharophila* ATCC 15946	NR_115049 (99)
KR779732	P112; P234; PB8	5	*Staphylococcus epidermidis* FDAARGOS_161	CP014132 (99)
MH424568	PxS	1	*Staphylococcus epidermidis* TWSL_19	KT184899 (100)
MH424569	PxS	1	*Staphylococcus caprae* OZK14	KT591476 (99)
MH424570	P112	1	*Staphylococcus* sp. JCE 11	LT899997 (100)
MH424571	PB8	1	*Staphylococcus* sp. clone 12L_53	KP183056 (99)
KR779733	PB8; PBo6S	2	*Ralstonia solanacearum* RSCM	CP025986 (99)
MH426745	PBo6S	1	*Ralstonia* sp. clone DVBSW_M180	KF755496 (100)
KR779734	PUSAS; PBo6S; P234; PB7'96; P112	11	*Cupriavidus metallidurans* Ni-2	CP026544 (100)
MH424572	PBo6S	1	*Cupriavidus* sp. EF11(2012)	JX912460 (99)
KR779735	PxS; P112	2	*Propionibacterium granulosum* JCM 6498	NR_113367 (99)
MH424575	P112	1	*Propionibacterium* sp. clone JPL-2_O14	FJ957593 (99)
KR779736	PB7'96; P112; PxS; P234; PB63S; Pbo6S	11	*Propionibacterium* sp. clone 12L_77	KP183061 (99)
KR779737	PxS; PB8; P234; P112	5	*Pseudomonas* sp. 09C 129	CP025261 (100)
KR779738	PB7'96	1	*Pseudomonas fluorescens* 2F9	KT695813 (100)
MH424593	PB7'96	2	*Pseudomonas fluorescens* PF85	MF838663 (100)
MH424580	PB8; PB63S	2	*Pseudomonas simiae strain* 4G1010	KY939757 (100)
MH424594	P112	1	*Pseudomonas fluorescens* L228	CP015639 (100)
MH424599	PB7'96	1	*Pseudomonas lurida* MYb11	CP023272 (100)
KR779740	P112; PB63S; PB7'96	4	*Acidovorax* sp. clone M_KL_81_14	KP967499 (100)
MH427201	PB63S; PBo6S	3	*Acidovorax* sp. clone CSC28	JN541150 (100)
KR779742	PUSAS	11	*Escherichia coli* DA33137	CP029579 (100)
MH427368	PUSAS	21	*Escherichia coli* 2012C-4502	CP027440 (100)
KR779743	PUSAS	31	*Escherichia coli* 2015C-3125	CP027763 (100)

Table I. Continued.

NCBI accessing number	Parent strain	Number of clones	The most similar strains in NCBI database	
			Strain name	Accessing number and similarity (%)
MH427381	PUSAS	1	*Escherichia coli* 2013C-3342	CP027766 (100)
KR779745	PxS	1	*Lactobacillus sakei* PR11	KX139193 (99)
MH427585	PxS	1	*Lactobacillus sakei* DS4	CP025839 (99)
MH427654	PB8	1	*Lactobacillus sakei* FAM18311	CP020459 (99)
KR779746	PxS	1	*Legionella* sp. L-29	AB856218 (98)
KR779747	PxS	1	*Finegoldia magna* JCM 1766	NR_113383 (99)
KR779748	PxS	1	*Sporosarcina psychrophila* DSM 6497	CP014616 (100)
KR779749	PBo6S	1	*Streptococcus pneumoniae* 11A	CP018838 (99)
KR779750	PB8	1	*Kocuria rhizophila* 3330	KP345929 (100)
KR779751	PB8	1	*Lactococcus garvieae* MJF010	MH057260 (100)
KR779752	P234	1	*Delftia lacustris* SH2	MH014970 (100)
KR779753	P234	1	*Pectobacterium carotovorum* subsp. *brasiliense* BC1	CP009769 (100)
KR779754	P234	1	*Oryzihumus leptocrescens* S32011-b	AB649006 (100)
KR779755	P234	1	*Tumebacillus* sp. 7B-408	KF441681 (99)
KR779756	P112	1	*Achromobacter mucicolens* OZK37	KT716268 (100)
KR779757	PB7'96	1	*Herbaspirillum* sp. WW2	KU495919 (100)
MH427999	P234; PBo6S; PB8; PB63S; PxS; PB7'96; P112	35	*Curvibacter* sp. clone Z2_KL_466-12	KP967473 (100)
MH428000	PB63S, PB7'96; P112	3	*Curvibacter* sp. clone CX 18.4	KX260804 (99)
MH428038	P234	1	*Acidobacteria* clone SEW_08_293	HQ598999 (99)
MH428102	PB7'96	1	*Acidobacteria* clone SEW_08_084	HQ598816 (99)
MH428220	P112	1	*Paenibacillus typhae* xj7	NR_109462 (99)
MH428377	P112	1	*Paenibacillus marchantiophytorum* R55	NR_148618 (99)
MH428379	P234	1	*Acinetobacter* sp. SWBY1	CP026616 (99)
MH428572	P234	1	*Acinetobacter towneri* MTCC11368T	KM070563 (99)
MH428659	PBo6S	1	*Streptomyces rishiriensis* JCM 4686	LC002811 (99)
MH428674	PBo6S	1	*Streptomyces* sp. 111013air4	KP262513 (99)
MH428833	PB7'96	1	*Sphingomonas* sp. CAU-S5	MF113252 (99)

USA) were transformed with the ligand, spread on LB medium with 50 ppm of kanamycin and inoculated for 24 h in 37°C. Randomly 48 colonies, grown on plates, representing every analyzed fungal strain were picked up and inoculated on LB medium with 50 ppm of kanamycin in 96-well plates. Clones were incubated for 24 h in 37°C and sent to LGC Genomics (Berlin) for plasmids sequencing using the Sanger method with primer M13-29R (5'-CAGGAAACAGCTATGACC-'3). Because of unusual results of PUSAS strain, the clones' identification procedure was repeated and 96 associated bacteria clones were sent for identification again. Sequences with the length of approximately 700 bp were analyzed using BLAST program in NCBI (USA) database. Sequences of clones identified to species or genus were deposited in NCBI database (Table I). Phylogenetic tree of the identified species or genus was build using the neighbor-joining method with maximum sequence difference set to 0.75. The tree was built in BLAST program and visualized in FigTree 1.4.3 (http://tree.bio.ed.ac.uk).

***Nif*H gene identification.** The identification of *Nif*H gene was performed by PCR with PolF and PolR primers according to the procedure described by Poly et al. (2001). The reaction mixture contained the tested DNA, 5 × Hot FIREPol Blend Master Mix buffer, and the primers mentioned above. Agarose gel electrophoresis of products was performed (agarose 2%, ethidium bromide 5%, electrophoresis time of 60 min, voltage 100 V). Products were visualized as described above. As a control, *Azospirillum barsieliense* 35Bb strain was used (Król and Perzynski 2005).

β-glucosidase gene identification. The identification of β-glucosidase gene was performed by PCR with bgluF and bgluR2 primers according to the procedure described by Canizares et al. (2011). The reaction mixture contained the isolated DNA, 5 × Hot FIREPol Blend Master Mix buffer, and the primers men-

tioned above. Products visualization was performed as described above.

β-xylanase gene identification. For the identification of the β-xylanase gene, PCR was performed with XynF and XynR primers according to the procedure described by Khandeparker et al. (2011). The reaction mixture contained the isolated DNA, 5×Hot FIREPol Blend Master Mix buffer and the primers mentioned above. Products visualization was performed as described above.

LMCO genes identification. The identification of LMCO (laccase-like multicopper oxidase) genes was performed by PCR with Cu1AFand Cu2R primers according to the procedure described by Kellner et al. (2008). The reaction mixture contained the isolated DNA, 5×Hot FIREPol Blend Master Mix buffer and the primers mentioned above. Products visualization was performed as described above.

Results

A number of small bacteria-like organisms were observed inside and outside of cells of all analyzed oyster mushroom strains. They were visible as small green or yellow-green rods. The microscopic image is shown in Fig. 1. The bacteria-like organisms were marked with white arrows. Several of the cells observed were motile inside of fungal hyphae what is presented in materials published online (PxS – https://youtu.be/G93Rm0tHIgg; P234 – https://youtu.be/pxApJvCshQ0; PB7'96 – https://youtu.be/JZdGXSyDdG4; PB63S – https://youtu.be/A4U4MtUeIiU). After 10 days of cultivation, a lot of small objects were observed growing deeply in the medium around and outside fungal mycelia of all inoculates (Fig. 2). Such growth was not observed on TSA medium without addition of Tween 20. Tween 20 is used usually as a surfactant to disperse cells in solutions. Some of the bacteria also use Tween 20 as a source of fatty acids. No objects were observed in control samples that were not inoculated with fungi. Similar results were described previously by Yara et al. (2006). Blocks of media containing objects were used for cells isolation. After 14 days of stationary cultivation in the liquid TSBt medium, a white precipitate was observed on flasks bottom for all oyster mushrooms strains analyzed. After centrifugation, this precipitate was used for DNA amplification protocol.

As a result of PCR performed with DNA obtained from precipitant and 799f and 1492r primers, a 700 bp product was obtained (Fig. 3). Product that size is characteristic only for bacterial DNA as it is a part of the bacterial 16S rRNA gene. For comparison, the reaction with maize DNA provided two bands: one of 1100 bp which is characteristic for plant mitochondrial DNA and second of size about 700 bp, characteristic for bacteria. The reason for this phenomenon is that maize cultivar used in experiment carry endophytic bacteria in its tissues (Pisarska and Pietr 2015).

The total number of 307 sequences was obtained as a result of the sequencing of plasmid clones. The detailed number of identified clones with the most

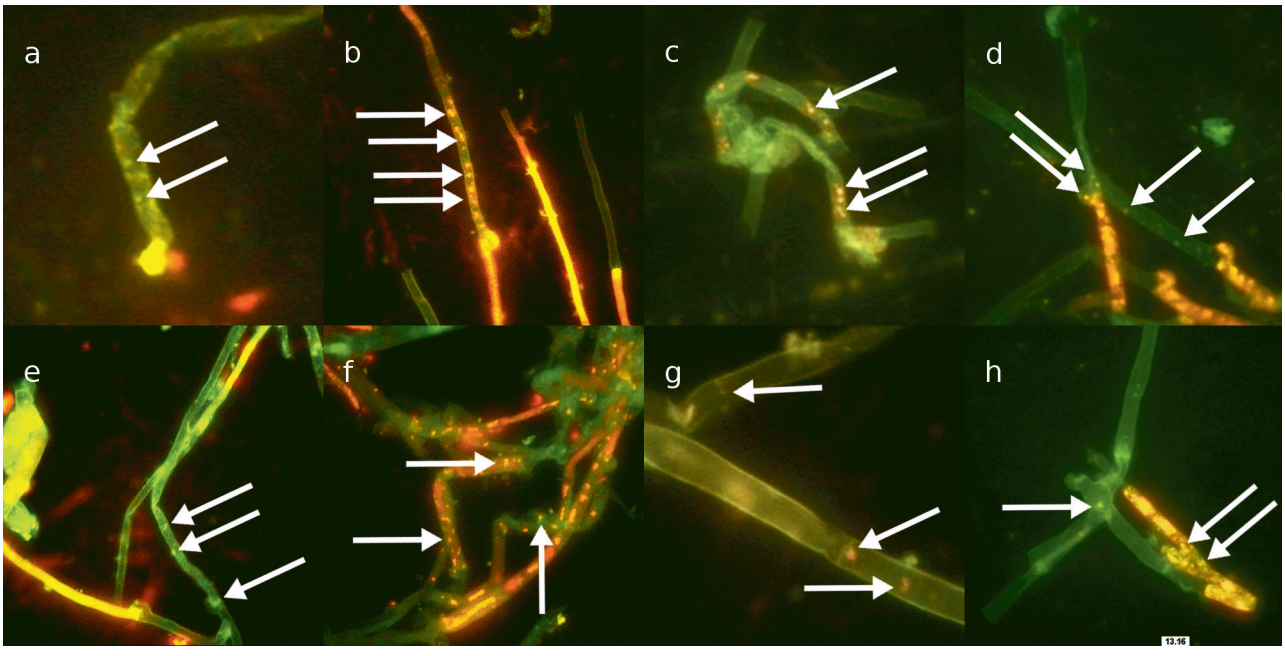


Fig. 1. Microscopic picture of analyzed oyster mushroom cells treated with fluorescent dyes under the fluorescent microscope. Strains: a) P234, b) PBo6S, c) PB8, d) P112, e) PB63S, f) PUSAS, g) PB7'96, h) PxS. Bacteria-like organisms, marked with white arrows, are visible as small green or yellow-green rods against fungal cells.

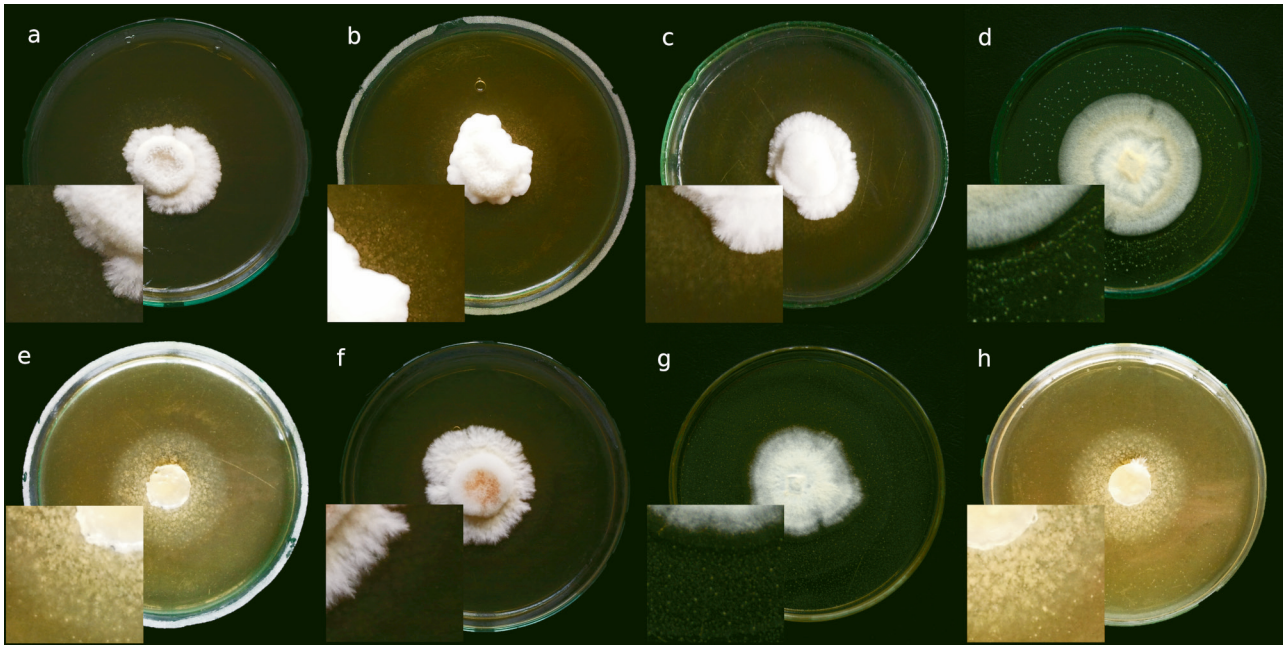


Fig. 2. Bacteria-like organisms microcolonies growing deeply in TSAt medium after 10 days long cultivation. Strains: a) P234, b) PBo6S, c) PB8, d) P112, e) PB63S, f) PUSAS, g) PB7'96, h) PxS.

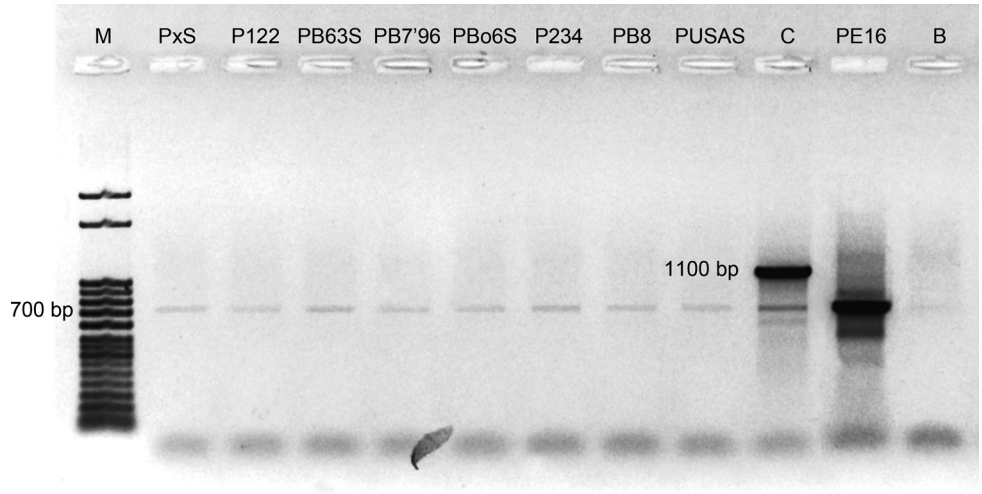


Fig. 3. Agarose gel electrophoresis showing products of PCR with 799f and 1942r primers of analyzed oyster mushroom, maize cv. Cyrkon (*Zea mays* L.) (line C) and bacterial strain *Pseudomonas fluorescens* PE16 (line PE16) DNA. The product of 700 bp is characteristic for bacterial 16S rRNA, the product of 1100 bp is characteristic for plant mitochondrial DNA. M – marker; B – blind control.

similar strains from NCBI database is presented in Table II. The species or genus was determined for 233 of them and for the remaining 74 sequences we were not able to identify higher taxonomic level than domena of *Bacteria*. All clones were identified only for PUSAS strain originated from the USA. Among other seven fungal strains, the unidentified clones occurred in the range from 7 to 15. Among tested strains of fungi, the lower biodiversity of bacteria was found for the strain PUSAS, which contained 64 clones closely related to *E. coli* and only two clones related to *Cupriavidus* genus. The only one genus, which occurred in all fungal strains analyzed but PUSAS, was *Curvibacter* and it was represented by 38 clones. *Propionibacterium* genus was found in six of eight fungal strains analyzed. It was not present only in PUSAS and PB8 strains. The total number of identified *Propionibacterium* strains was 14. Another genus found in six of *P. ostreatus* strains was *Cupriavidus*. It occurred in a total number of 13 clones in all fungal strains except PxS and PB63S. Bacteria belonging to genus *Pseudomonas* were also identified in a number of 12 clones from six fungal strains (except PUSAS and PBo6S). Genus *Pelomonas* was identified as nine clones from five fungal strains: P234, PB7'96, PB8, PxS, and P112. Bacteria from genus *Paracoccus* in a number of six were found in almost all of *P. ostreatus* strains from

Table II
Number and identification of clones obtained from analyzed *P. ostreatus* strains.

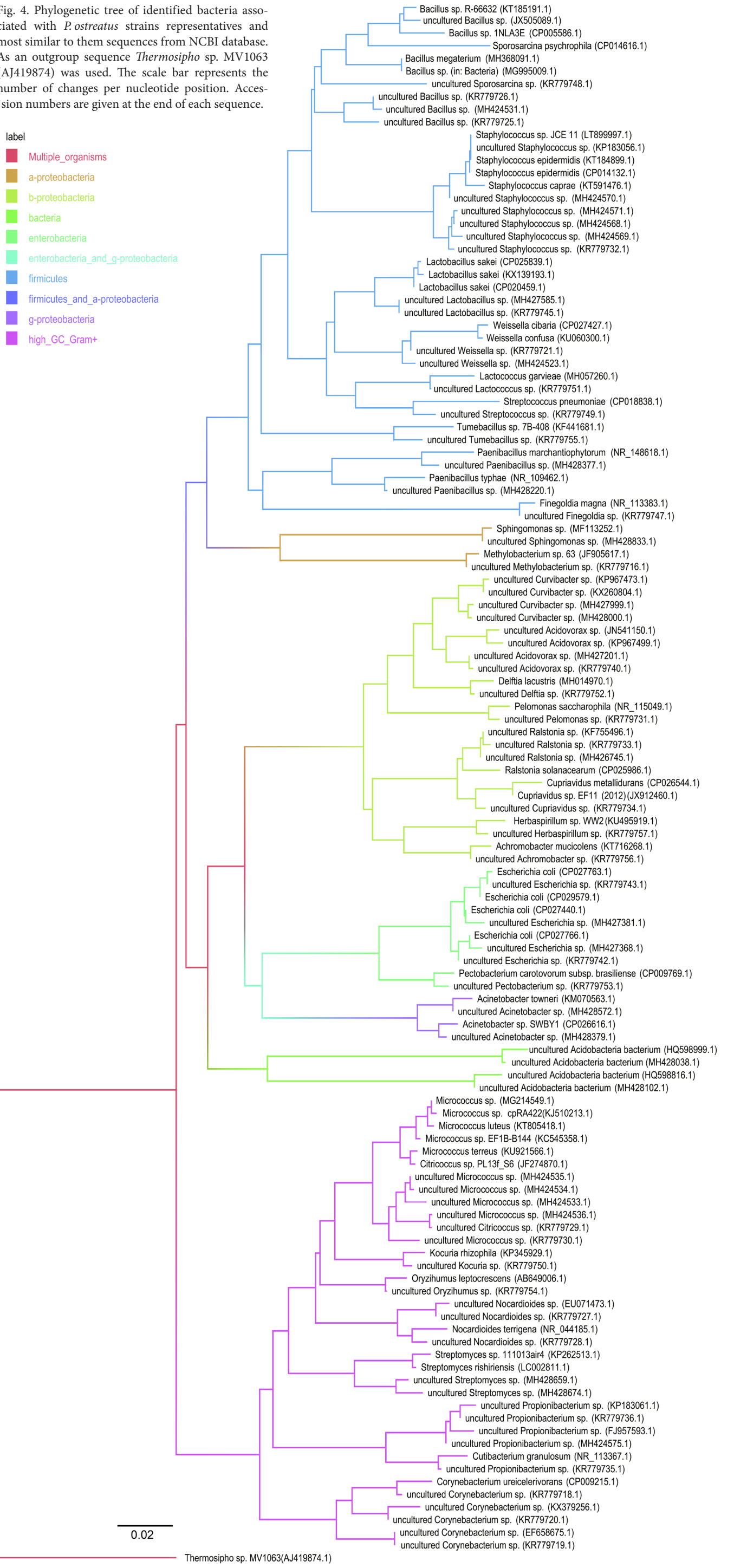
Genus	Similarity based on 700 bp sequence of the 16S rRNA gene		Number of clones	Origin *P. ostreatus* strains							
				USA	Japan		Poland				
	The most closely related strain in NCBI	NCBI accession number (% of similarity)		PUSAS	PB8	PB7'96	PBo6S	P234	PxS	PB63S	P112
Escherichia	*E. coli* DA33137	CP029579 (100)	64	11	0	0	0	0	0	0	0
	E. coli 2012C-4502	CP027440 (100)		21	0	0	0	0	0	0	0
	E. coli 2015C-3125	CP027763 (100)		31	0	0	0	0	0	0	0
	E. coli 2013C-3342	CP027766 (100)		1	0	0	0	0	0	0	0
Curvibacter	*Curvibacter* sp. clone Z2_KL_466-12	KP967473 (100)	38	0	6	8	4	6	4	2	5
	Curvibacter sp. clone CX 18.4	KX260804 (99)		0	0	1	0	0	0	1	1
Propionibacterium	*Propionibacterium* sp. clone 12L_77	KP183061 (100)	14	0	0	1	1	2	2	2	3
	P. granulosum JCM 6498	113367 (99)		0	0	0	0	0	1	0	1
	Propionibacterium sp. clone JPL-2_O14	FJ957593 (99)		0	0	0	0	0	0	0	1
Bacillus	*Bacillus* sp. CHORDb1	MG995009 (100)	13	0	2	0	1	2	0	0	3
	Bacillus sp. clone D	JX505089 (99)		0	0	0	0	0	0	0	2
	Bacillus sp. 1NLA3E	CP005586 (99)		0	0	0	0	1	0	0	0
	Bacillus sp. R-66632	KT185191 (99)		0	0	1	0	0	0	0	0
	Bacillus megaterium Y103	MH368091 (99)		0	1	0	0	0	0	0	0
Cupriavidus	*C. metallidurans* Ni-2	CP026544 (100)	12	2	2	1	3	2	0	0	2
	Cupriavidus sp. EF11(2012)	JX912460 (99)		0	0	0	1	0	0	0	0
Pseudomonas	*Pseudomonas* sp. 09C 129	CP025261 (100)	12	0	2	0	0	1	1	0	1
	P. fluorescens PF85	MF838663 (100)		0	0	2	0	0	0	0	0
	P. simiae 4G1010	KY939757 (100)		0	1	0	0	0	0	1	0
	P. fluorescens L228	CP015639 (100)		0	0	0	0	0	0	0	1
	P. fluorescens 2F9	KT695813 (100)		0	0	1	0	0	0	0	0
	P. lurida MYb11	CP023272 (100)		0	0	1	0	0	0	0	0
Staphylococcus	*S. epidermidis* FDAARGOS_161	CP014132 (99)	9	0	1	0	0	2	0	0	2
	S. epidermidis TWSL_19	KT184899 (100)		0	0	0	0	0	1	0	0
	S. caprae OZK14	KT591476 (99)		0	0	0	0	0	1	0	0
	Staphylococcus sp. JCE 11	LT899997 (100)		0	0	0	0	0	0	0	1
	Staphylococcus sp. clone 12L_53	KP183056 (100)		0	1	0	0	0	0	0	0
Pelomonas	*P. saccharophila* ATCC 15946	NR_115049 (99)	9	0	2	1	0	2	3	0	1
Acidovorax	Acidovorax sp. clone M_KL_81_14	KP967499 (100)	4	0	0	1	0	0	0	1	2
	Acidovorax sp. clone CSC28	JN541150 (100)	3	0	0	0	1	0	0	2	0
Weissella	*W. cibaria* BM2	CP027427 (99)	6	0	0	1	2	0	1	0	0
	W. confusa SM10	KU060300 (100)		0	0	0	0	0	0	0	2
Micrococcus	*M. luteus* JGTA-S5	KT805418 (100)	6	0	0	0	0	1	0	0	0
	Micrococcus sp. cpRA422	KJ510213 (100)		0	1	0	0	0	0	0	0
	Micrococcus sp. EF1B-B144	KC545358 (99)		0	0	0	0	0	1	0	0
	Micrococcus sp. strain CAU1456	MG214549 (99)		0	0	0	0	0	0	0	1
	M. terreus IHBB 9339	KU921566 (99)		0	0	0	0	1	1	0	0
Paracoccus	*Paracoccus* sp. clone SL36	HQ264096 (100)	6	0	0	0	1	1	2	1	0
	P. yeei TWCC 57946	LC371258 (100)		0	0	1	0	0	0	0	0
Nocardioides	*N. terrigena* DS-17	NR_044185 (99)	5	0	0	2	2	0	0	0	0
	Nocardioides sp. clone EHFS1_S02a	EU071473 (99)		0	0	0	0	0	1	0	0
Ralstonia	*R. solanacearum* RSCM	CP02598 (99)	3	0	1	0	1	0	0	0	0
	Ralstonia sp. clone DVBSW_M180	KF755496 (100)		0	0	0	1	0	0	0	0

Table II. Continued.

Genus	Similarity based on 700 bp sequence of the 16S rRNA gene		Number of clones	Origin *P. ostreatus* strains							
				USA	Japan		Poland				
	The most closely related strain in NCBI	NCBI accession number (% of similarity)		PUSAS	PB8	PB7'96	PBo6S	P234	PxS	PB63S	P112
Corynebacterium	*C. ureicelerivorans* IMMIB RIV-2301	CP009215 (99)	3	0	0	0	1	0	0	0	0
	Corynebacterium sp. clone YHSS1	EF658675 (100)		0	0	0	1	0	0	0	0
	Corynebacterium sp. clone OD-12	KX379256 (100)		0	1	0	0	0	0	0	0
Lactobacillus	*L. sakei* FAM18311	CP020459 (99)	3	0	0	0	1	0	0	0	0
	L. sakei PR11	KX139193 (99)		0	0	0	0	0	1	0	0
	L. sakei DS4	CP025839 (99)		0	0	0	0	0	1	0	0
Acidobacteria	*Acidobacteria* clone SEW_08_293	HQ598999 (99)	2	0	0	0	0	1	0	0	0
	Acidobacteria clone SEW_08_084	HQ598816 (99)		0	0	1	0	0	0	0	0
Paenibacillus	*P. typhae* xj7	NR_109462 (99)	2	0	0	0	0	0	0	0	1
	P. marchantiophytorum R55	NR_148618 (99)		0	0	0	0	0	0	0	1
Acinetobacter	*Acinetobacter* sp. SWBY1	CP026616 (99)	2	0	0	0	0	1	0	0	0
	A. towneri MTCC11368T	KM070563 (99)		0	0	0	0	1	0	0	0
Streptomyces	*S. rishiriensis* JCM 4686	LC002811 (99)	2	0	0	0	1	0	0	0	0
	Streptomyces sp. 111013air4	KP262513 (99)		0	0	0	1	0	0	0	0
Legionella	*Legionella* sp. L-29	AB856218 (98)	1	0	0	0	0	0	1	0	0
Finegoldia	*F. magna* JCM 1766	NR_113383 (99)	1	0	0	0	0	0	1	0	0
Sporosarcina	*S. psychrophila* DSM 6497	CP014616 (100)	1	0	0	0	0	0	1	0	0
Streptococcus	*S. pneumoniae* 11A	CP018838 (99)	1	0	0	0	1	0	0	0	0
Kocuria	*K. rhizophila* 3330	KP345929 (100)	1	0	1	0	0	0	0	0	0
Lactococcus	*L. garvieae* MJF010	MH057260 (100)	1	0	1	0	0	0	0	0	0
Delftia	*D. lacustris* SH2	MH014970 (100)	1	0	0	0	0	1	0	0	0
Pectobacterium	*P. carotovorum* subsp. *brasiliense* BC1	CP009769 (100)	1	0	0	0	0	1	0	0	0
Oryzihumus	*O. leptocrescens* S32011-b	AB649006 (100)	1	0	0	0	0	1	0	0	0
Tumebacillus	*Tumebacillus* sp. 7B-408	KF441681 (99)	1	0	0	0	0	1	0	0	0
Achromobacter	*A. mucicolens* OZK37	KT716268 (100)	1	0	0	0	0	0	0	0	1
Herbaspirillum	*Herbaspirillum* sp. WW2	KU495919 (100)	1	0	0	1	0	0	0	0	0
Citricoccus	*Citricoccus* sp. PL13f_S6	JF274870 (99)	1	0	0	0	0	0	1	0	0
Sphingomonas	*Sphingomonas* sp. CAU-S5	MF113252 (99)	1	0	0	1	0	0	0	0	0
Number of different clones	X	X	X	5	15	16	17	18	17	8	17
Number of genus	X	X	32	2	9	13	13	15	13	5	12
Number of unidentified clones of *Bacteria*	X	X	74	0	6	10	14	10	15	7	12

Poland (except P112). The remaining clones identified were as follows: *Bacillus* – 13 clones (in P234, PBo6S, PB8, P112), *Staphylococcus* – nine clones (in P234, PB8, PxS, P112), *Acidovorax* – seven clones (in PB7'96, PBo6S, PB63S, P112), *Weisella* – six clones (in PB7'96, PBo6S, PxS, P112), *Micrococcus* – six clones (in P234, PB8, PxS, P112), *Nocardioides* – five clones (in PB7'96, PBo6s, PxS), *Ralstonia* – three clones (in PBo6S, PB8), *Corynebacterium* – three clones (in PBo6S, PxS), *Lactobacillus* – three clones (in PBo6S, PxS), *Acidobacteria* – two clones (in P234, PB7'96), *Paenibacillus* – two clones (in P112), *Acinetobacter* – two clones (in P234), *Streptomyces* – two clones (in PBo6S), *Legionella* – one clone (PxS), *Finegoldia* – one clone (PxS), *Sporosarcina* – one clone (PxS), *Streptococcus* – one clone (PBo6S), *Kocuria* – one clone (PB8), *Lactococcus* – one clone (PB8), *Delftia* – one clone (P234), *Pectobacterium* – one clone (P234), *Oryzihumus* – one clone (P234), *Tumebacillus* – one clone (P234), *Achromobacter* – one clone (P112), *Herbaspirillum* – one clone (PB7'96),

Fig. 4. Phylogenetic tree of identified bacteria associated with *P. ostreatus* strains representatives and most similar to them sequences from NCBI database. As an outgroup sequence *Thermosipho* sp. MV1063 (AJ419874) was used. The scale bar represents the number of changes per nucleotide position. Accession numbers are given at the end of each sequence.



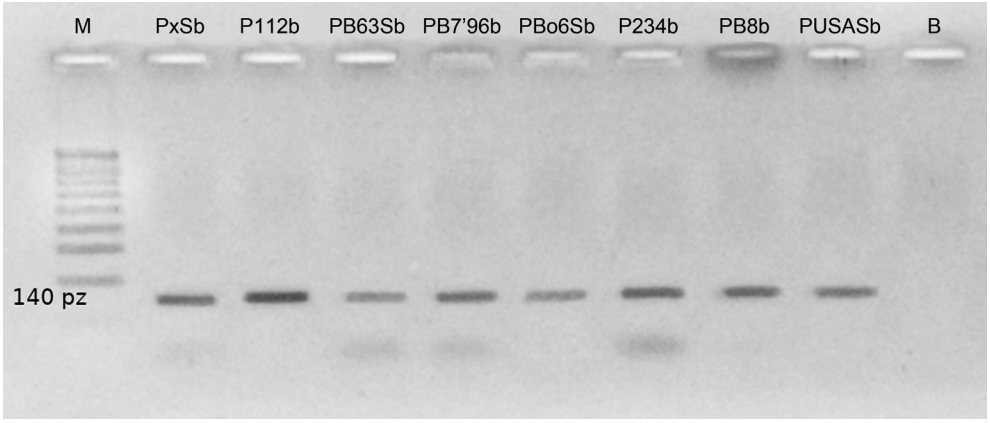


Fig. 5. Agarose gel electrophoresis showing products of PCR with Cu1AF and Cu2R primers and analyzed oyster mushroom DNA. Visible 140 bp band represents amplified LMCO genes. M – marker; B – blind control.

Citrococcus – one clone (PxS), *Sphingomonas* – one clone (PB7'96). The detailed number of identified clones with the most similar strains from NCBI database is presented in Table II. Phylogenetic tree of representatives of all identified genotypes with most similar sequences from NCBI database is presented in Fig. 4. As an out-group sequence, the *Thermosipho* sp. MV1063 (AJ419874) was used. The detailed number of identified clones with the most similar strains from NCBI database is presented in Table II.

The PCR reactions designed for detection of *Nif*H, β-glucosidase and β-xylanase genes gave no product for all analyzed fungal strains. However, LMCO genes were detected (Fig. 5). LMCO is a complex of enzymes, which include laccases (EC 1.10.3.2) among others.

Discussion

Yara et al. (1999, 2006) reported that bacteria or bacteria-like organisms could interact with only one strain of *P. ostreatus*. However, authors did not confirm the endocellular character of these bacteria, they described them only as bacteria associated with *P. ostreatus* G2 hyphae. Using fluorescent microscopy technique, which was described by Bianciotto et al. (2000), we found bacteria associated with all eight tested strains of *P. ostreatus* originating from different geographic zones (Japan, Poland, and the USA). Every specimen was prepared from living cells and their movement was possible to observe under a microscope. These observations and lack of growth of isolated organisms without the presence of host on microbiological media strongly suggested the endosymbiotic character of the observed bacteria. This hypothesis was also strongly supported by the fact that cultivation of the fungal strains on media with wide range of antibacterial antibiotics (ampicillin, neomycin, gentamicin, penicillin G, streptomycin, polymyxin B, kanamycin, ciprofloxacin, detreromycin, tobramycin (200 ppm), meropenem, and ceftadizine (30 ppm), data unpublished) did not eradicate bacteria from fungal mycelia. Similar techniques used for the isolation of bacteria from five strains of *Lentinula edodes* not revealed the presence of associated bacteria (data unpublished). Due to the fact that it was not possible to fully distinguish between endosymbiotic and associated bacteria among the isolates tested we decided to use in this study the term "bacteria associated with". Literature of subject shows the presence of one or two species of bacteria associated with *P. ostreatus*. Yara et al. (2006) described bacteria associated with one strain of *P. ostreatus* belonging to *B. cepacia* complex, which is complex of at least 20 different species from *Burkholderia* genus (LiPuma 2005). In this work, we report the presence of bacteria related to at least 34 different genera living in association with eight *P. ostreatus* strains originating from different geographical regions. It was noticeable that bacteria from genus *Curvibacter* were isolated from almost all analyzed fungal strains. The only one strain that did not contain these bacteria was a PUSAS strain. This strain also was the only one associated with *E. coli* bacteria what is difficult to explain due to lack of knowledge of history of this strain, The bacteria from the *Curvibacter* genus were reported endosymbionts of *Oryza sativa* roots (Singh et al. 2006), similarly as an uncultured bacterium from *Chlorella* cultures (Otsuka et al. 2008) and tomato rhizosphere (Lioussanne et al. 2010) and the bacteria associated with *Hydra*, which serve as a protective factor against pathogen infections (Fraune et al. 2015). Bacteria of genera *Bacillus* and *Pseudomonas* are well-known plant growth-promoting bacteria due to competitive and antagonistic activity versus several pathogens (Compant et al. 2010). The occurrence of *Curvibacter*, *Bacillus* and *Pseudomonas* bacteria associated with *P. ostreatus* could be related to the known lower susceptibility of this edible mushroom

when compare to *Agaricus bisporus* (J.E. Lange) Imbach. *Gigasporacea* sp. endosymbionts were described as N_2 fixing bacteria (Bianciotto et al. 2003). However, our study did not show the presence of nitrogenase reductase genes using *Pol*F and *Pol*R primers; however, the occurrence of clones similar to *Cupriavidus* suggest that these bacteria could be N_2-fixers associated with oyster mushroom. Strains of *Cupriavidus taiwanensis*, previously were isolated from nodules of *Mimosa*, can nodulate also legumes and fix N_2. However, the *nif*H gene is only distantly related to other alphaproteobacterial rhizobial strains (Gyaneshwar et al. 2011), what was probably a reason that used primers were not specific in this case. The bacteria associated with *P. ostreatus* could also influence their hosts' capability for lignocellulolytic substrates utilization. PCR reactions with specific primers for β-glucosidase, β-xylanase genes did not reveal their presence, which suggests that these bacteria did not play important role in degradation of polysaccharides. However, the presence of LMCO genes was identified in this study. Laccases are a group of enzymes able to use a variety of substrates, including lignin (Rekuc et al. 2006). This suggests that although oyster mushroom-associated bacteria were able to produce neither β-glycosidase nor β-xylanase, they still could be able to support the lignocellulolytic activity of the strains tested due to laccase activity.

The origin of identified *P. ostreatus*-associated bacteria, their phylogenesis, and relationship with hosts were difficult to determine. In the natural environment, the oyster mushroom is a saprophyte, which develops on dead trunks and deciduous trees. Occasionally, it can develop in places of cuts on living trees. Such a variety of hosts may be the reason for the biodiversity of bacterial associations. Different non-pathogenic species probably inhabited mycelium of *P. ostreatus* during colonization of various hosts.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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