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ORIGINAL ARTICLE

Kocuria Bacterial Isolates from Radioactive Springs of Jáchymov spa (Joachimsthal) as Sources of Polyunsaturated Fatty Acids

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Abstract Four bacterial isolates, which produced polyunsaturated fatty acids (PUFA), were isolated from water samples of radioactive springs collected from Jáchymov spa. Jáchymov (Sankt Joachimsthal) is a city in northwestern Bohemia, where Marie and Pierre Curie isolated radium in 1898 from the mineral uraninite. To date, four springs (Agricola, Behounek, C1, and Curie) have been used for spa purposes, that is for the treatment of nervous and rheumatic disorders by constantly produced radioactive gas radon (^{222}Rn) dissolved in the water. The radioactivity reaches 24 kBq/L. Using 16S rRNA gene sequence analysis, all four isolates were identified as members of the genus *Kocuria*, with two isolates designated 208 and 401 affiliated with *Kocuria kristinae*, while isolates 101 and 301 most likely with *K. rhizophila*. The content of fatty acids in polar lipids was determined by gas chromatography–mass spectrometry (GC–MS) and two PUFA, that is arachidonic and eicosapentaenoic, were identified. The position of double bonds was confirmed by GC–MS of 3-pyridylcarbinol (formerly picolinyl) esters. We assume that all four isolates of *Kocuria* produce PUFA to increase the stability of cell membranes, which may be impaired by the reaction of the reactive oxygen species. These can arise, for example, because of α radiation during ^{222}Rn decay.

Keywords Arachidonic and eicosapentaenoic acids · Jáchymov spa · *Kocuria sp.* · Polyunsaturated fatty acids · Radioactive springs

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Abbreviations

ARA	arachidonic acid
DHA	docosahexaenoic acid
EFA	essential fatty acids
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
FAS	fatty acid synthase
GC–MS	gas chromatography–mass spectrometry
LB	Luria–Bertani
PCA	plate count agar
PCR	polymerase chain reaction
PKS I	polyketide synthase I
PUFA	polyunsaturated fatty acid
R2A	Reasoner's 2A
ROS	reactive oxygen species
SIM	selected-ion monitoring
TAG	triacylglycerols
TIC	total ion current
TSA	tryptone soya agar
YPD	yeast extract-peptone-dextrose

Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Lipids are important components of the membranes of both prokaryotic and eukaryotic organisms and play a significant role not only as a source of energy (Sijtsma and de Swaaf, 2004), for instance n-3 and n-6 polyunsaturated fatty acids (PUFA), but also participate in various physiological processes, influencing architecture, dynamics, phase transition, and permeability of membranes (Tapiero et al., 2002).

In higher eukaryotes including mammals and humans, PUFA are precursors of known physiologically active metabolites such as eicosanoids, for example prostaglandins, prostacyclins, thromboxanes, and leukotrienes, and are, therefore, known as fatty acids essential to human organ development during life.

So far, two basic pathways for the biosynthesis of PUFA have been described. The first and more familiar includes fatty acid synthase (FAS) followed by desaturation and elongation of palmitic (oleic) acids to form PUFA. In addition, an alternative pathway for PUFA biosynthesis in both prokaryotic and eukaryotic organisms uses polyketide synthase I (PKS I) (Abd Elrazak et al., 2013).

Mammals cannot synthesize essential fatty acids (EFA) because they lack the enzymes required to synthesize *de novo* PUFA and are thus dependent on external sources (diet). At present, sea fish and some vegetable oils are the main sources; unfortunately, sea pollution has reached such an extent that even fish oil is contaminated by undesirable pollutants (Wardrop et al., 2016). As a result, alternative sources such as bacteria, fungi, or algae are being sought. Because of using these resources, the potential number of consumers is expanding to vegetarians and vegans.

In addition, the presence of PUFA in the form of phospholipids in bacteria is far more advantageous for consumers than PUFA in triacylglycerols (TAG), which are essentially all plant and fish oils, because in various clinical applications (Kidd, 2007; Wijendran et al., 2002) it has been proven that phospholipids are better utilized than TAG. It has been reported that PUFA-producing bacteria are most commonly found in deep sediment and cold water (Yoshida et al., 2016). PUFA production is associated with high pressure and low temperature.

Bacteria of the genus *Kocuria* (family Micrococcaceae, order Actinomycetales, class Actinobacteria) are gram-positive cocci often found in tetrads or irregular clusters. To date, more than 18 species of *Kocuria* were identified based on the 16S rRNA gene studies (Kandi et al., 2016). Bacteria of this genus were isolated from, for example, freshwater fish gut microbiota (Jami et al., 2015), marine sediment sample (Bala et al., 2012), saline and alkaline soils (Wang et al., 2015), unconsolidated subsurface sediments (Crocker et al., 2000), saline soil (Tang et al., 2009), cattail leaves collected in river (Kovacs et al., 1999), seawater collected on the coast of Korea (Seo et al., 2009), marine sediment from the Indian Ocean (Jiang et al., 2015), a deep-sea hydrothermal plume (Zhang et al., 2017), and marine sediment (Kim et al., 2004).

The content of fatty acids in *Kocuria* is very similar to other bacteria of phylum Actinobacteria. Major fatty acids are iso-15:0 and anteiso-15:0 fatty acids, minor ones are iso-14:0, 14:0, iso-16:0, 9–16:1, 16:0, anteiso-17:1, iso-17:1, iso-17:0, anteiso-17:0, 18:0, and 9–18:1. Fatty acids with two double bonds were identified only exceptionally, for example 18:2 (Kim et al., 2004; Kovacs et al., 1999; Seo et al., 2009; Wang et al., 2015), always using the SHERLOCK Microbial Identification System. No PUFA have been identified for any *Kocuria* bacteria, although the PKS I complex has been identified (Jami et al., 2015; Palomo et al., 2013).

In our study, bacteria of genus *Kocuria* were collected from water samples of radioactive springs of Jáchymov spa. The town Jáchymov (Sankt Joachimsthal) is a city in northwestern Bohemia, where Marie and Pierre Curie isolated radium from the mineral uraninite in 1898. To date, four springs (Agricola, Behounek, C1, and Curie) have been used for spa purposes, that is for the treatment of nervous and rheumatic disorders by constantly produced radioactive gas radon (^{222}Rn) dissolved in the water. The radioactivity reaches 24 kBq/L. The presence of fatty acids in our bacteria was also confirmed and the predominant fatty acids were (%): iso-15:0 (16.6); anteiso-15:0 (65.4); and anteiso-17:1n-9 (2.8). They were identified using gas chromatography–mass spectrometry (GC–MS) as methyl esters and as 3-pyridyl carbonyl derivatives (formerly picolinyl esters). 20:4n-6 (arachidonic acid [ARA]) and 20:5n-3 (eicosapentaenoic acid [EPA]) fatty acids formed during cultivation were present at up to 3.4% total fatty acids. As part of the search for new sources of food supplements, see the two grants mentioned in Acknowledgments, new and prospective PUFA resources were explored. Bacteria, unlike the fish and vegetable oils mentioned above, are not subject to ever greater environmental contamination, including for example microplastics. Bacteria are also well cultivated, as demonstrated in the genus *Streptomyces*, belonging to the same order of Actinomyceteles as the genus *Kocuria.* Streptomycetes produce antibiotics in dozens of thousands of liters, so the biotechnology is well managed (Smith, 1986). For these reasons, we see bacterial-based biotech production of PUFA in the future as very promising.

Materials and Methods

Collection and Cultivation

Samples were collected from four springs (Agricola, Behounek, C1, and Curie) in Joachimstahl, Czech Republic. Sterile

50 mL Falcon tubes were used for the sampling. Each tube was either empty or contained 2.5 or 5 mL of sterile Luria–Bertani (LB) or yeast extract-peptone-dextrose (YPD) broth. The spring water was filled to 25 mL or 50 mL to achieve a 10-fold dilution of the media. The samples were then transported back to laboratory and incubated on rotary shakers (150 rpm) at temperatures set at 30 °C (samples from Agricola, C1 and Curie) or 37 °C (samples from Behounek). The cultivation temperatures matched the water temperature of the springs. After 7 and 30 days of cultivation in Falcon tubes, the samples were inoculated onto different agar plates and spread with an L-shaped bent glass rod. The agar media used in this study were prepared from the respective filtered spring water.

After 7 days of cultivation in Falcon tubes, a 0.1 mL aliquot from each of the sample was inoculated to the surface of the agar medium prepared by dissolving glucose (0.2%) and bacteriological agar (2.3%) in filtered spring water. The plates were incubated at 30 °C (Agricola, C1, and Curie) or 37 °C (Behounek) for 1 week.

After 30 days of cultivation in Falcon tubes, each sample was diluted to achieve a final cell concentration of 1.10^3 cells/mL. A 0.1 mL aliquot from each of the diluted sample was inoculated to the surface of the agar medium. The agar media were: Reasoner's 2A (R2A) agar; lactate (0.5%) agar medium; and pure noble agar (1.8%) medium. The plates were incubated at 30 °C (Agricola, C1, and Curie) or 37 °C (Behounek) for 1–3 weeks.

Several distinct colonies with different morphologies were transferred from the above agar plates to plate count agar (PCA) plates and incubated at 30 or 37 °C for 24 h for further isolation and purification.

Finally, for the lipid analysis, four bacterial isolates (designated 101, 208, 301, and 401) were transferred from PCA plates to tryptone soya agar (TSA) for 48 h and using sterile Eppendorf tubes (Schoeller Pharma, Prague, Czech Republic). The cultivation temperature for isolate 208 was 37 °C and for the remaining three isolates 30 °C. Cell mass was frozen at −70 °C and lyophilized.

DNA Extraction and 16S rRNA Amplification

DNA Extraction

Thermal lysis (Wald et al., 2015) was used for the extraction of bacterial DNA. Briefly, the isolates were cultivated on PCA plates for 24 h at 30 or 37 °C (according to the temperatures of the spring waters). Following the period of growth, a few colonies of each isolate were collected in microtubes with 50 µL of water for molecular biology (Sigma-Aldrich, USA) and incubated for 20 min at 98 °C. Afterward, the cell debris was pelleted for 5 min at 10,000 × *g*. The supernatant with DNA was removed and stored at −20 °C for further use.

16S rRNA Gene Amplification and Analysis

Polymerase chain reaction (PCR) amplification of 16S rRNA genes was performed in a final volume of 15 µL and final concentration of reagents: 0.3 µM forward primer 8F 5'-AGAGTTTGATCMTGGCTCAG-3', (Sigma-Aldrich); 0.3 µM reverse primer 1492R 5'-GYTACCTTGTTACGACTT-3′ (both Lane, 1991), (Sigma-Aldrich); 0,02 U/µL KAPA Hifi HotStart Ready mix 2x (KAPA Biosystems, USA); and 1 ul of DNA-containing supernatant after thermal lysis. The amplification program conditions were: initial denaturation for 5 min at 95 °C; 25 cycles of denaturation (20s at 98 °C), annealing (15 s at 56 °C) and extension (40s at 72 °C); and final extension for 5 min at 72 °C. Resultant PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research, USA) following the manufacturer's recommendation. The DNA so obtained was diluted to a final concentration of 40 ng/µL. Some 2.5 µL of each PCR product was mixed with 0.5 nmol of 8F, 1492R, or 1068R 5'-CTGRCGRCRRC CATGCA- 3' (Leewis et al., 2016) primers and 2.5 µL of water for molecular biology. Capillary sequencing was performed commercially at Eurofins Genomics company. The resulting sequences were edited in MEGA 7 (Kumar et al., 2016) and identified by EZBioCloud (Yoon et al., 2017) and RDP Seqmatch (Cole et al., 2014) tools.

Isolation of Lipids

The extraction procedure was based on the method of Bligh and Dyer (1959). Briefly, the lyophilized cells were suspended in a chloroform-methanol mixture (2:1) for 30 min with stirring, after which chloroform and water were added and the insoluble material was separated by centrifugation. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35 cc (Waters; with 10 g of normal-phase silica), and nonpolar lipids were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (9:1). Polar lipids were eluted from the cartridge by a mixture of 50 mL of methanol acidified with 0.1% formic acid. The eluate of polar lipids was evaporated and the oil samples were further hydrolyzed.

Fatty Acid Methyl Ester (FAME) Analysis

Fatty acids were released from polar lipids, see above, by acid hydrolysis (2 M HC1, 100 °C, 2.5 h), extracted with

hexane, and methylated with 10% (w/v) methanolic BF_3, (80 °C, 10 min). GC–MS of the FAME mixture was done on a Finnigan 1020 B in the EI mode. Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. The structures of FAME were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAME (Supelco, Prague) (Rezanka et al., 2012; Vancura et al., 1988).

3-Pyridylcarbonyl Esters of Fatty Acids

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate FAME (~0.5 mg) in dry dichloromethane (1 mL) were added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

A fatty acid 3-pyridylcarbonyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column (Supelco, Czech Republic) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500.

Results

Bacterial Isolation, Taxonomy, and Phylogeny

Altogether, 131 bacterial isolates were obtained from Joachimstahl spring samples. Out of these isolates, 4 were identified as members of the genus *Kocuria* and tested for their ability to produce PUFA. Bacterial isolates investigated in this study were obtained using the following culture conditions: isolate *Kocuria* sp. 101 was obtained from R2A agar plates inoculated with samples from Falcon tubes containing 50 mL of a 10-fold diluted LB broth; isolate *Kocuria* sp. 208 from R2A agar plates inoculated with samples from Falcon tubes containing 50 mL of a 10-fold diluted YPD broth; isolate *Kocuria* sp. 301 from agar plates containing only glucose inoculated with samples from Falcon tubes containing solely the respective spring water; and isolate *Kocuria* sp. 401 from agar plates containing only glucose inoculated with samples from Falcon tubes containing 25 mL of a 10-fold diluted YPD broth.

Using 16S rRNA gene sequence analysis, all four isolates were identified as members of the genus *Kocuria*, with the closest type strains shown in Table 1. Isolates *Kocuria* sp. 208 and 401 had the same closest type strain *Kocuria kristinae* NBRC 15354^T and their placement in the phylogenetic tree (Fig. 1) indicated they are very likely of common origin. In addition, their colonies appeared similar in terms of morphology; they were lightly pink in color, raised, and had undulate margins. Isolates *Kocuria* sp. 101 and 301, although they shared the closest type strain (*Kocuria rhizophila* DSM 11926^T), differed in their position in the phylogenetic tree (Fig. 1) as well as in morphology. Isolate *Kocuria* sp. 101 formed irregular, umbonate colonies with undulate margins. This isolate also produced bright yellow pigments. Isolate *Kocuria* sp. 301 formed light yellow colonies, which were raised and had regular borders.

Identification of Fatty Acids

The content of fatty acids contained in polar lipids of *Kocuria* genus is summarized in Table 2. Total fatty acids ranged in 2–3% of dry matter, with a PUFA content of 2–3% of total fatty acids. The content of EPA was the highest in isolate 401 obtained from the Curie spring and represented 616 μg per 1 g of dry matter. All four isolates contained predominantly branched fatty acids with an odd number of carbon atoms, mainly iso-15:0 and anteiso-15:0, with a minor proportion (in percentage units of total fatty acids) of other fatty acids.

The time of the FAME analysis was extended, see Fig. 2, so that, under identical conditions, three standards of PUFA were eluted from the column, that is ARA, EPA, and docosahexaenoic (DHA) acids. Retention times (tR = 19.210

Table 1 The closest type strain of the isolates investigated based on 16S rRNA gene analysis

Isolate (accession no.)	The closest type strain based on 16S rRNA gene analysis[a]	Source
101 (MH538969)	*Kocuria rhizophila* DSM 11926^T	water spring Agricola
208 (MH539149)	*Kocuria kristinae* NBRC 15354^T	water spring Behounek
301 (MH539151)	*Kocuria rhizophila* DSM 11926^T	water spring C1
401 (MH539150)	*Kocuria kristinae* NBRC 15354^T	water spring Curie

[a]The pairwise similarity between 16S rRNA genes was >99% in all cases.

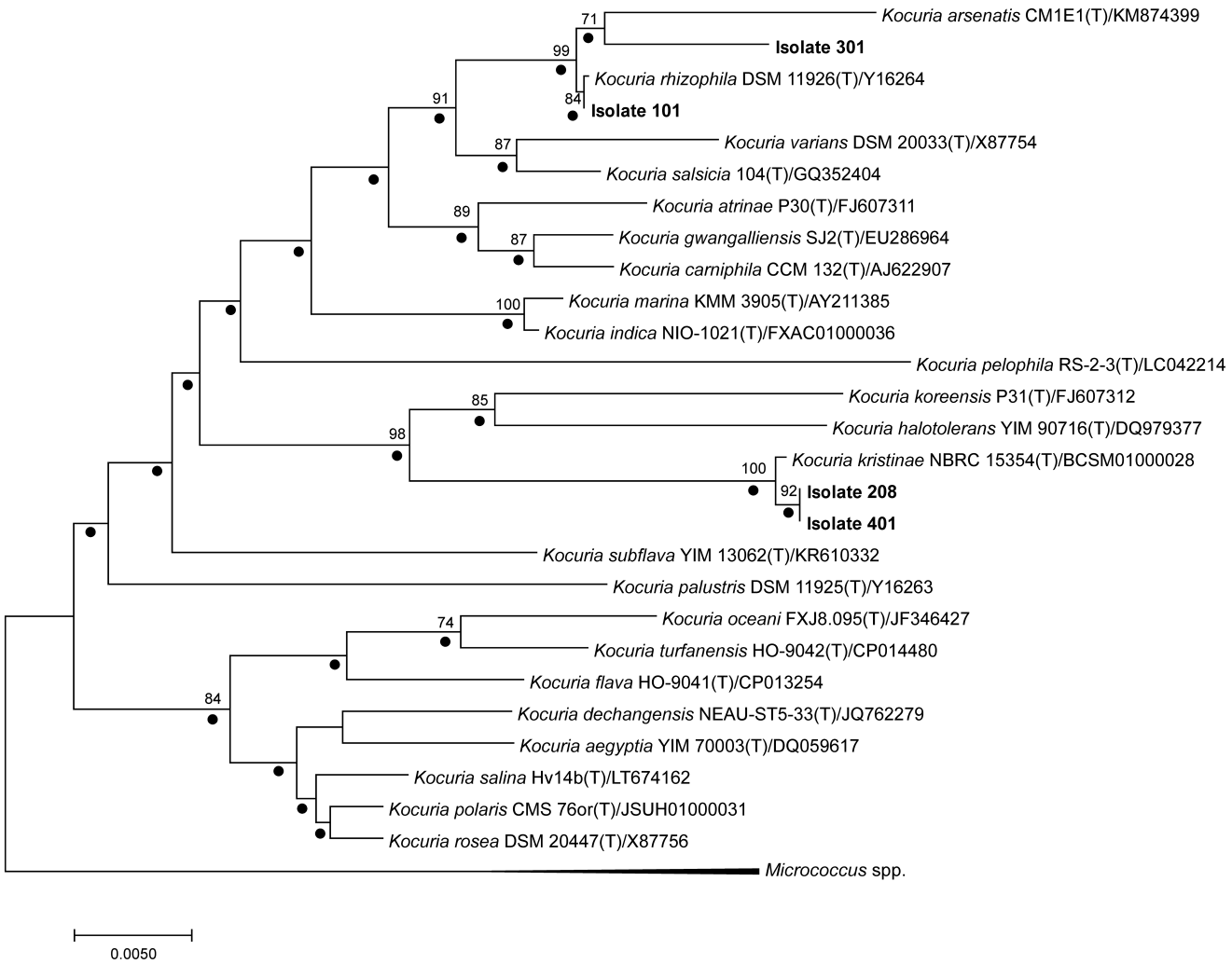

Fig. 1 Evolutionary relationships of the isolates 101 (accession no.MH538969), 208 (accession no. MH539149), 301 (accession no. MH539151), and 401 (accession no. MH539150) and their closest relatives from the genus *Kocuria.* The phylogenetic tree was constructed in MEGA7 (Kumar et al., 2016) using secondary structure-based alignment. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). All positions with less than 95% site coverage were eliminated; there were 1356 positions in total in the final dataset. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap test (1000 replicates) was used to test tree topology (Felsenstein, 1985); only bootstrap values ≥70 are indicated. Nodes obtained also by maximum-likelihood are indicated with dots. The analysis involved 29 nucleotide sequences, two *Micrococcus* sp. were included as the outgroup (Genbank accessions no. CP001628.1 and NZ_FPCG01000029.1). The bar represents 0.0050 substitutions per site

and 19.285 min, respectively) of commercially available standards (ARA and EPA) overlap with the retention times of two peaks as shown in Fig. 2. Based on the mass spectra of two peaks at retention times 19.210 and 19.285 min, we identified two PUFA, namely ARA and EPA. Fig. 3 shows the same GC–MS record in which the sensitivity of the mass spectrometer (total ion current [TIC]) was increased 10x.

Preliminarily, the position of the double bonds was based on the fragmentation of FAME, because n-6 FAME are known to contain an abundant ion at *m/z* 150, while n-3 contain an ion at *m/z* 108 (Fellenberg et al., 1987). Because the methyl esters of PUFA have molecular ion $[M]^+$ of either very low abundance or even absent, 3-pyridylcarbinol (formerly picolinyl) esters of fatty acids were prepared. Their GC–MS analysis was performed in selected-ion monitoring (SIM) mode (see Fig. 4). It is clear from this figure that two peaks were separated and identified at *m/z* 333.2, where the structure of branched pentadecanoic acids, namely iso-15:0 and anteiso-15:0, was confirmed by the mass spectra. The mass spectra of two ions in SIM mode at *m/z* 395.3 and 393.3 are shown in Figs. 5 and 6. These two mass spectra include the corresponding structural formulas showing the cleavage of the 3-pyridylcarbinol esters in the mass spectrometer. The mass spectra of

Table 2 Fatty acids identified in four isolates of Kocuria sp. (see Table 1)

tR[a]	FAME	Isolate 101	Isolate 208	Isolate 301	Isolate 401
8.525	Iso-13:0	1.2	0.1	0.3	0.2
8.663	Anteiso-13:0	0.6	0.2	0.2	0.3
10.153	Iso-14:0	1.8	0.8	2.1	2.1
10.725	14:0	0.9	0.9	0.5	1.1
11.769	Iso-15:0	16.6	19.3	17.2	12.3
11.897	Anteiso-15:0	65.4	62.5	67.2	68.7
12.044	15:1[b]	0.2	0.0	0.0	0.9
12.355	15:0	0.0	0.6	0.3	0.3
13.395	Iso-16:0	1.1	0.8	1.0	1.0
13.591	16:1n-7	2.0	3.1	2.0	2.3
13.985	16:0	0.9	1.1	0.8	0.9
14.941	Anteiso-17:1n-9	2.8	3.9	2.3	2.8
15.027	Iso-17:0	0.5	0.3	0.5	0.4
15.159	Anteiso-17:0	2.7	3.9	1.6	1.8
16.615	18:2n-6	0.1	0.0	0.1	0.2
16.659	Iso-18:0	0.3	0.0	0.4	0.2
16.763	18:1n-9	0.2	0.4	0.3	0.4
17.245	18:0	0.6	0.3	0.7	0.7
19.210	**20:4n-6**[c]	**0.2**	**0.6**	**0.4**	**0.7**
19.285	**20:5n-3**	**1.9**	**1.2**	**2.1**	**2.7**

[a]Retention time (min).

[b]Position of the double bond was not determined.

[c]Bold values represent PUFA.

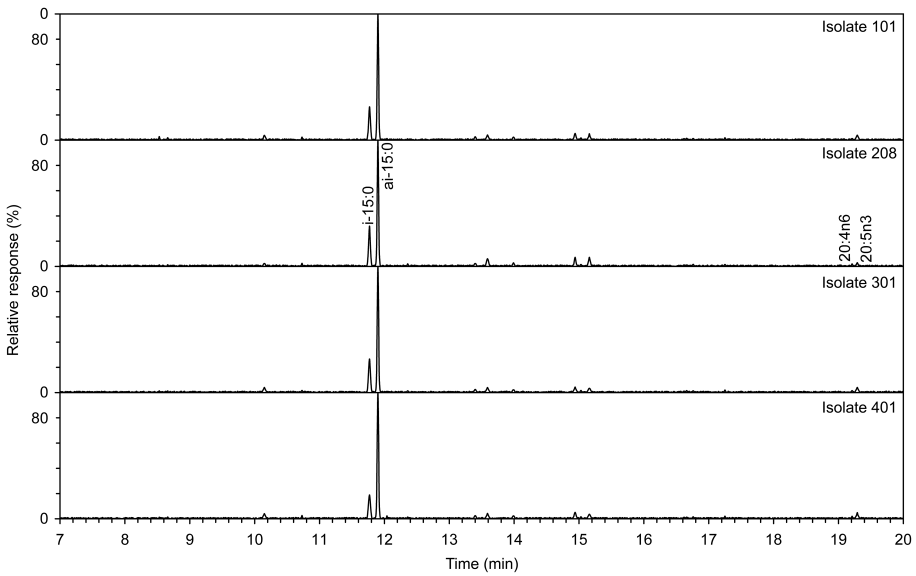

Fig. 2 Gas chromatography–mass spectrometry (GC–MS) chromatogram (total ion current, TIC) of the four isolates of genus *Kocuria* sp.

these derivatives are characterized by abundant ions at *m/z* 92, 108, 151 (McLafferty rearrangement), and 164. The structure can be determined based on ions in which the gap of 28 Da changed to a gap of 26 Da (—CH_2—CH_2— to —CH=CH—).

An example is a 3-pyridylcarbinol ester of EPA (Fig. 5) with a gap of 26 Da between *m/z* 364.3 and 338.3, which locates a double bond at position 17, while the gap between *m/z* 324.3 and 298.3 locates the double bond at position 14. Other gaps at *m/z* 284.2–258.2, 244.2–218.2, and

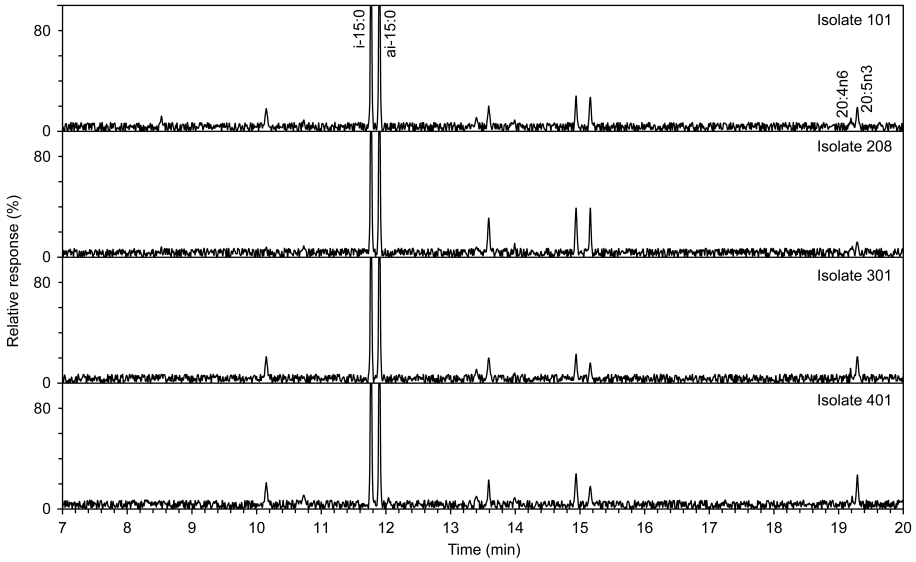

Fig. 3 Gas chromatography–mass spectrometry (GC–MS) chromatogram (total ion current, TIC) of the four isolates of genus *Kocuria* sp. The same records, only the sensitivity was 10 times higher

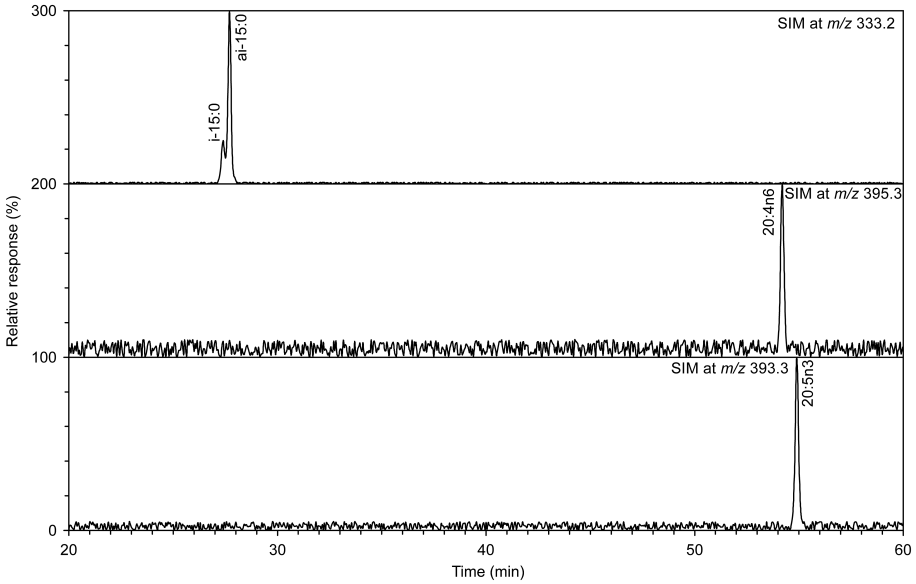

Fig. 4 Selected ion monitoring (SIM) of the following values: 333.2 Da (iso or anteiso 15:0), 395.3 Da (arachidonic acid, ARA), and 393.3 Da (eicosapentaenoic acid, EPA)

204.1–178.1 unambiguously confirm the structure 20:5n-3 (5, 8, 11, 14, and 17–20:5). Among other things, our mass spectrum is very similar to the mass spectrum published by Christie (2018). The structure of ARA was proven to be similar, see Fig. 6.

Discussion

Most bacteria that produce PUFA have been isolated from marine sources and consist of a narrow group of gram-negative γ-*Proteobacteria*, for example genera *Shewanella*, *Photobacterium*, *Moritella*, *Colwellia*, and *Vibrio* (Shulse and Allen, 2011a). They are characterized by being psychrophilic (psychrotrophic) or piezotrophic (barophilic). However, studies on the presence of *pfa* genes encoding PUFA synthase proteins and their homologs proved their presence also in terrestrial bacteria including myxobacterial genera *Sorangium* and *Aetherobacter* (Gemperlein et al., 2014) as well as gram-positive bacteria of the genus *Rhodococcus* (Shulse and Allen, 2011b). The question of the function of PUFA in the membranes of bacteria has already

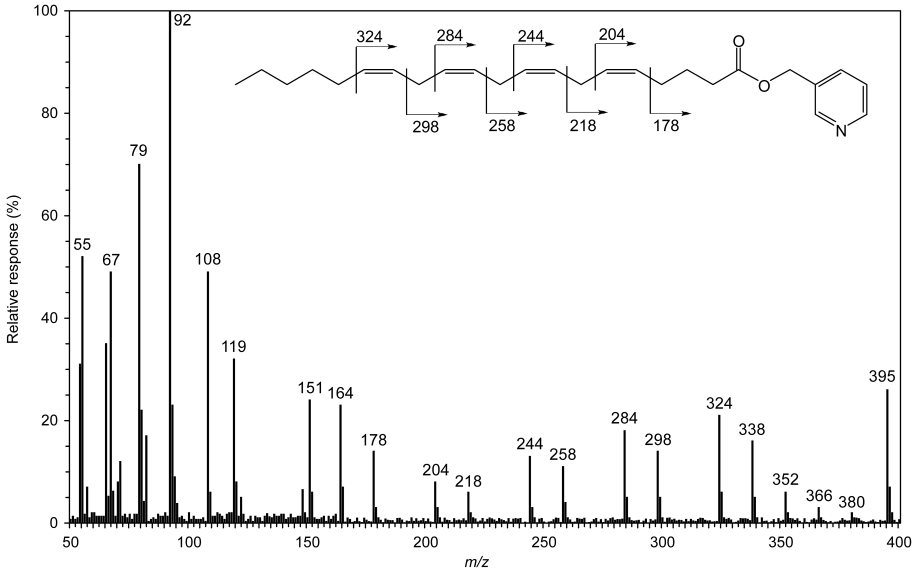

Fig. 5 Mass spectrum of 3-pyridylcarbonyl (picolinyl) ester of 20:4n-6 acid (ARA). For explanation of values, see the text

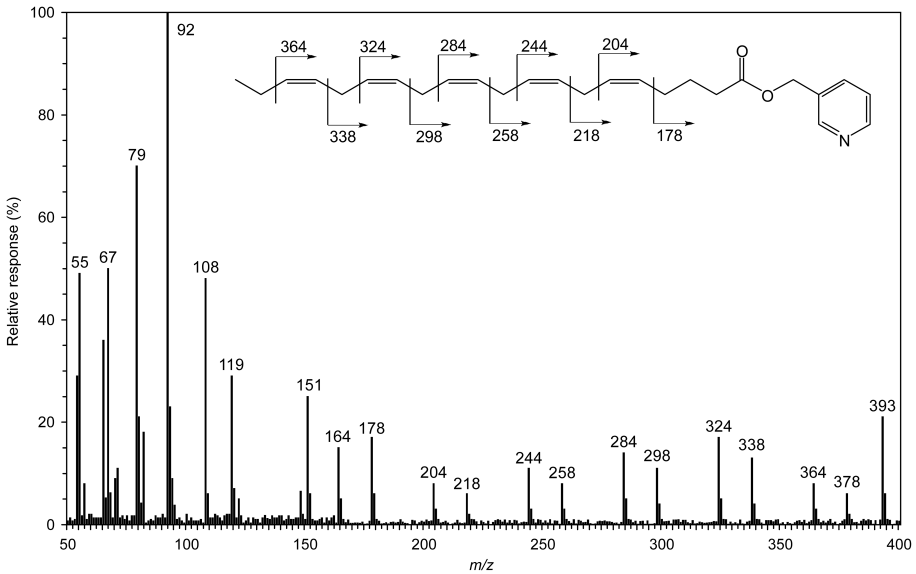

Fig. 6 Mass spectrum of 3-pyridylcarbonyl (picolinyl) ester of 20:5n-3 acid (EPA). For explanation of values, see the text

been discussed (Yoshida et al., 2016). For example, in *Shewanella piezotolerans*, the EPA content rises to more than four times the control level with increasing pressure (0.1 to 20 MPa) and decreasing temperature (20 to 4 °C). The bacteria of the gram-positive genus *Kocuria* that we studied come from groundwater located approximately 1 km below the Earth's surface where the water pressure is about 100 MPa. However, because the cultivation was carried out at normal atmospheric pressure (~0.1 MPa), there can be no conclusions in this regard and it can be assumed that our *Kocuria* bacteria produce PUFA for other reasons, for example, their antioxidant functions.

All species of the genus *Kocuria* (see Table S1), contain the major anteiso-15:0 acid. The abundance of other fatty acids varies; however, based on the published data, the other but less-abundant fatty acids are iso-14:0, 14:0, iso-16:0, 16:0, anteiso-17:0 acids, and others. Minor acids include, for example, iso-15:1, 15:1, 16:1, iso-17:1, and other acids that were identified only in some species of *Kocuria.* These include the two identified PUFA, namely ARA and EPA.

16S rRNA gene sequences of the isolates Kocuria sp. 208 and 401 are identical and the isolates are likely affiliated with *Kocuria kristinae* (Fig. 1 and Table 1), but it is likely that these isolates are different ecotypes

because they were obtained from different water springs (Behounek and Curie) with different temperatures, which were sustained during the cultivation period (37 and 30 °C, respectively). The cultivation temperatures had most likely an effect on the presence of PUFA in both isolates, because the EPA and ARA levels are higher in isolate *Kocuria* sp. 401 grown at lower temperatures, see Table 2. The effect of growth temperature on fatty acid composition was well reported in many studies (e.g. Marr and Ingraham, 1962; Ray et al., 1971; Wang et al., 2016).

With only one exception, to our current knowledge, the position of the double bonds has not been directly determined. Individual PUFA were identified only based on GC–MS analysis of retention times and mass spectra of methyl esters. Only Watanabe et al. (1997) determined the positions of double bonds directly by GC–MS of pyrrolidide derivatives.

The production of PUFA by *Kocuria* may be related to the fact that radioactive decay in all three series (Thorium, Uranium and Actinium) gives rise to radon (Rn) that is an α emitter. The water of all four springs (Agricola, Behounek, C1, and Curie) in the Svornost mine contains a large amount of radon. Radiochemical decay of ^{222}Rn (half-life of 3.82 days) creates α particles that cause water radiolysis to form highly reactive products of the type of hydroxyl radicals, hydrogen radicals, and also possibly hydrogen peroxide.

Radon-containing springs are found in many places around the world, but research of bacterial community in them is only in its beginning. The first mention of the bacteria living in these springs was published in 1973 (Yoshinaka et al., 1973) in the hot spring in Misasa, Japan. Later, this bacterium (*Arthrobacter radiotolerans*) was renamed *Rubrobacter radiotolerans* (Kausar et al., 1997) by the use of 16 s rRNA gene analysis. Together with the development of DNA sequencing (16 s rRNA gene), the number of identified bacteria has increased. In the South Australia's region, there is Paralan Hot Spring with water whose radioactivity reaches up to thousands Bq/L, in which bacteria of various genera, such as *Nitrospira*, *Hydrogenophilus*, *Desulforomonas*, and so on. have been identified. Unfortunately, most of them were identified only as clone(s) with GenBank accession number(s) (Anitori et al., 2002, 2004).

Many clones of the Nitrospirae, Bacteroidetes, and Planctomycetes (Weidler et al., 2007) have been isolated from the springs in Bad Gastein near Salzburg (Austria), whose radioactivity is many hundreds of Bq/L. Even though the sequences were compared with sequences stored in GenBank, the authors were unable to determine a more detailed taxonomic classification. The spring of Wettinquelle (Bad Brambach) situated on the Czech Republic-Germany border is the geographically closest to the Jáchymov Spa. Radioactivity is very similar to that found in Jáchymov (27 kB/L in Wettinquelle versus 24 Bq/L in Behounek spring). Many tens of clones have been identified (the sequences are also stored in GenBank). Moreover, in the case of some of the clones, also other genera were identified, such as *Gallionella*, *Thiobacillus*, or *Sulfuricurvum* (Wagner et al., 2007).

Finding bacteria of the genus *Kocuria* in radioactive water is not uncommon. Bacteria of the genus *Micrococcus roseus* (now *Kocuria rosea*) (Brooks and Murray, 1981) resistant to radioactive radiation have been described as early as 1981. One of the possible mechanisms of the antioxidant function of PUFA is the formation of a hydrophobic interface between the phospholipid bilayer composed of both PUFA and saturated and monounsaturated fatty acids (Rajamoorthi et al., 2005). This hydrophobic layer then forms a barrier for hydrophilic compounds, including H_2O_2.

Unfortunately, all relevant publications are older than 10 years, when there were a much lower number of bases in GenBank (https://www.ncbi.nlm.nih.gov/genbank/statistics/). In our publication, we compared the four isolate sequences with a database containing much more sequences. Therefore, it was possible to identify bacteria with certain accuracy up to a species with 100% accuracy on the genus—*Kocuria.*

In conclusion, awareness of the nutritional and pharmaceutical values of PUFA in human health has resulted in the usage of sea fish oils as food supplements. However, because of marine pollution, there is a growing demand for alternative sources of PUFA, especially those of microbial origin. PUFA are mostly detected in marine bacteria that inhabit cold and deep seawater. Based on 16S rRNA gene sequence-based phylogenetic analysis, four bacterial isolates (*Kocuria* sp. 101, 208, 301 and 401) from Jáchymov spa (Joachimstahl) radon containing spring water were affiliated with the genus *Kocuria.* All four strains produced PUFA, that is ARA and EPA, in amounts of a few percent of total fatty acids. PUFA are known to be reactive toward reactive oxygen species (ROS) and free radicals because of the weak C-H bonds at the *bis*-allylic group (Halliwell and Gutteridge, 2015), and, therefore, PUFA located in cell membrane are rather stable against exogenous ROS (Nishida et al., 2006).

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